DETECTION OF *CRYPTOSPORIDIUM* AND *E. COLI* USING FLUORESCENT *IN SITU* HYBRIDIZATION AND SOLID PHASE LASER CYTOMETRY

by

Susan Cameron Broadaway

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APPROVAL

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Susan Cameron Broadaway

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Barry H. Pyle

Approved for the Department of Microbiology

Mark Jutila

Approved for The Graduate School

Dr. Ronald W. Larsen

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Susan Cameron Broadaway

May 2013

DEDICATION

This is dedicated to my mother, Rosemary Cameron, who, at 60, wanted to start her own business but didn’t because she was thought she was too old.

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If not for the encouragement of Barry Pyle, who insisted that I should become a graduate student, I would have never begun, much less finished this degree. Thanks to the other members of my committee, Anne Camper and Sandra Halonen for their guidance, Betsey Pitts for her help with the DIC microscope and Metamorph program and Al Parker for the statistical analyses. I appreciate the Crow Environmental Health Steering Committee who provided a real world project to test my method and Mari Eggers who facilitated that relationship. Thanks to the operators at the Crow Agency Water Treatment Plant for allowing access for sampling and to Steve Hamner and Tami Old Coyote who took some samples for me. Thanks also to Kien Lim, who assisted with the tyramide signal amplification procedure.

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ABSTRACT

Cryptosporidium parvum is a protozoal pathogen transmitted through water by the fecal-oral route as oocysts. Because the oocysts are more resistant to environmental stresses than the bacteria conventionally used as indicators of fecal contamination, they can be present in water when indicator organisms, such as *E. coli,* are not found. In addition, because they are resistant to chlorine, they can pass from source water through water treatment into drinking water systems. The EPA method for detection of *Cryptosporidium* oocysts consists of identifying oocysts with fluorescently labeled antibodies, staining with 4',6-diamidino-2-phenylindole and examining slides with epifluorescent microscopy and differential interference contrast microscopy. This protocol is labor intensive and subject to technician error. A new method was developed for the rapid detection of *Cryptosporidium parvum* oocysts using fluorescent *in situ* hybridization (FISH) and the Scan*RDI*, a solid phase laser cytometer. Optimization of the FISH protocol for use with the Scan*RDI* was done with *E. coli* cells and known *Cryptosporidium* oocysts as a model. Source water and treated drinking water from the water treatment plant at Crow Agency on the Crow Indian Reservation in Montana was collected over the course of a year and concentrated using the EPA protocol for collection of oocysts. The samples were then examined for *Cryptosporidium* oocysts using both the Scan*RDI* method and the standard US EPA method. The combination of FISH for labeling *Cryptosporidium* and the Scan*RDI* for examination results in significantly higher numbers of *Cryptosporidium* detected as well as greater ease in identification. A statistical comparison was done that determined there was no correlation between the number of *E. coli* cells found in the water samples and the number of *Cryptosporidium* oocysts present. Additionally, although not tested on environmental samples, the FISH/Scan*RDI* method allowed for simultaneous detection of *Cryptosporidium parvum* oocysts and *E. coli* cells on the same membrane filter. Membranes were incubated before hybridization, hybridized concurrently with a *Cryptosporidium* specific probe and a probe specific for *E. coli,* followed by detection for both organisms with the Scan*RDI.*

INTRODUCTION

Present methods used to determine if drinking water is microbially contaminated rely on detection of indicator organisms such as *E. coli* that show if there has been fecal contamination rather than direct detection of pathogens themselves (1). Ideally the absence of the indicators should always signal the absence of pathogens. However, this is not always the case. Because pathogens and indicator bacteria react differently to environmental stresses, pathogens can be present in drinking water even when no indicator bacteria have been detected (2, 3). Additionally, because the methods for detection of indicator organisms rely on culture techniques, it can take days before indicator bacteria can be identified. The goal of this study was to find a rapid method to detect multiple pathogens directly from water.

In order to request a grant to improve the Crow Agency water treatment plant, the Crow Environmental Health Steering Committee was interested to know if the source water for the plant contained *Cryptosporidium* oocysts. *Cryptosporidium parvum* is a waterborne protozoal pathogen often associated with cattle, causing sometimes severe diarrhea in humans. In collaboration with the committee, *Cryptosporidium* was chosen as the pathogen of interest for this project.

Because it is important to be able to detect the presence of small numbers of pathogens from drinking water the Scan*RDI* was the primary detection method. The Scan*RDI* is a solid phase laser cytometer. It is similar to a flow cytometer in that it uses lasers to detect fluorescently labeled particles but, rather than detection in a fluid stream, particles are immobilized on a membrane filter held by a specialized filter holder. After

the instrument scans the entire surface of the membrane, which takes about 3 minutes, the holder can be moved to an epifluorescent microscope and the scanner software directs a motorized stage to each of the particles. This allows the user to verify whether each particle is the labeled target or if it is debris. The Scan*RDI* can detect one fluorescent particle on a filter giving it a very low detection limit. Flow cytometers need at least 100 cells for efficient detection (4) and there has to be at least 10,000 to 100,000 targets present to be able to reliably enumerate cells with an epifluorescent microscope.

Fluorescent *in situ* hybridization (FISH) is a technique that uses oligonucleotide probes with the sequences specific for a target organism to hybridize to portions of the ribosomal RNA (5). These probes can be labeled with fluorophores with the emission spectrum that can be detected by the Scan*RDI*. There have been previous studies using the Scan*RDI* to detect cells labeled by (FISH) (6-8). This project sought to extend that method in order to detect *Cryptosporidium* in water with the Scan*RDI*.

E. coli was chosen in this study to optimize the FISH technique on the membrane filter for use with the Scan*RDI* due to the availability of information on *in situ* hybridization of *E. coli* (7, 9-14) and the relationship of this organism to drinking water safety (1). Included in optimization were prefixation incubation with antibiotics to increase number of ribosomes, different permeabilization methods, different probe types and concentrations, different hybridization times and temperatures, all with the goal of using this method for *Cryptosporidium*. "Fluorescent *in situ* Hybridization of *E. coli"* describes this optimization experimentation.

As described in chapter titled "Fluorescent *in situ* Hybridization of *Cryptosporidium*", once the standard protocol for *E. coli* was developed, optimization of

FISH for use with *Cryptosporidium* was done using control oocysts. It was determined that the protocol developed for *E. coli* was satisfactory for identifying *Cryptosporidium* with FISH for detection by the Scan*RDI*.

The following chapter, "Detection of *Cryptosporidium* from the Environment" describes the use of the EPA recommended protocol for concentration of *Cryptosporidium* from the Little Big Horn River, the source for the Crow Agency drinking water treatment plant. Once the oocysts were concentrated, the sample was examined for the presence of *Cryptosporidium* using both the FISH/Scan*RDI* protocol and the EPA recommended method of detection; fluorescent antibodies combined with a nucleic acid stain and differential interference contrast microscopy.

In keeping with the goal of detection of multiple organisms at the same time, the results of combining *E. coli* and *Cryptosporidium* on the same membrane are shown in "Detection of *Cryptosporidium* and *E. coli*".

The final chapter discusses the results of the project and describes some problems and possible solutions for improving the concentration, not only of *Cryptosporidium* from water, but also other pathogens at the same time.

FLUORESCENT *IN SITU* HYBRIDIZATION OF *E. COLI*

Introduction

Fecal contamination of drinking water is a source of disease-causing viruses, bacteria, and protozoa. *E. coli* has been routinely used as an indicator of fecal contamination in drinking water for many years. Descriptions of the culture based methods approved for use in the United States are available in Standard Methods for the Examination of Water and Wastewater (1). Because determination of the microbiological safety of drinking water as described in Standard Methods (1) requires at least overnight incubation, and because the safety of drinking water is crucial and time sensitive, more rapid methods using molecular techniques to identify the presence of *E. coli* have been explored. A review of these methods can be found in Rompré et. al. (8).

The Scan*RDI* (AES Chemunex) is a solid-phase laser cytometer that is able to scan the entire surface of a 25 mm diameter membrane filter using a 488-nm laser (4, 15-18). Fluorescence emitted by particles on the membrane is recorded and screened by computer software using several discriminant parameters. The fluorescent events determined by the software application to be the target organisms are enumerated and the location of each particle on the membrane is mapped. The membrane is then placed on a motorized stage of an epifluorescent microscope and the Scan*RDI* software directs the microscope stage to each fluorescent particle, allowing the operator to confirm the discrimination between debris and target organisms. This system allows the detection and verification of even a single fluorescent particle on a membrane filter. The threshold of

detection and the discrimination parameters can be manipulated to provide more precise identification of organisms depending on the labeling procedure.

Fluorescent *in situ* hybridization (FISH) is a method of identifying individual microorganisms to genus or species using short oligonucleotides that pass through the cell wall and bind to the rRNA. Use of FISH to detect *E. coli* has been extensively studied (7, 9-14). *E. coli* was chosen in this study to optimize the FISH technique on the membrane filter for use with the Scan*RDI* due to the availability of information on *in situ* hybridization of *E. coli* and the relationship of this organism to drinking water safety. Several different probes were examined with different fluorescent labels in order to ascertain the probe that would provide the brightest signal.

Originally an oligonucleotide probe designated ECO 541 was chosen for this work as it was reported to provide a bright signal when compared to other potential 16S probes for *E. coli*. It has been postulated that the brightness of the signal was likely due to accessibility of the target sequence in the ribosome that allowed the FISH probe to bind easily (12). The methods developed by Baudart, et. al (6), that described the use of a 16S rRNA probe for Enterobacteriaceae detected by the Scan*RDI* , were adapted for use with the ECO probe and *E. coli*. Experiments showed that this probe coupled with horse radish peroxidase followed by the tyramide signal amplification protocol could be used with the Scan*RDI*, but this probe was not specific for *E. coli*, in fact it also probed *Aeromonas hydrophila*, used a as control organism. Another probe designated Colinsitu was shown by the Regnault group to specifically label *E. coli*, *Escherichia fergusonii* and *Shigella spp.* (11), but was in a fairly inaccessible region of the ribosome (12) and thus was unlikely to provide an adequately strong fluorescent signal for the Scan*RDI* detection

when cells were probed using FISH. Fuchs, et. al., (19) found that by adding unlabeled 'helper' probes, that bind to positions flanking the labeled probe target region in the ribosome, it was possible to increase the fluorescent signal in otherwise inaccessible region. Baudart and Lebaron in a report published in 2010 (7), used the Colinsitu probe labeled with fluorescein along with unlabeled helper probes to detect *E. coli* with the Scan*RDI*. We adapted the methods described by Baudart, used a 2 to 4 hour prefixation incubation with the antibiotic nalidixic acid in R2A broth, a 90 minute hybridization with the combination of Colinsitu probe labeled with horse radish peroxidase and unlabeled helper probes in the hybridization buffer followed by amplification of the signal with tyramide. By adjusting the application software of the Scan*RDI* to detect the FISH probed cells, we found good correlation between plate counts and enumeration with the Scan*RDI*.

Methods

Bacterial Strains

An environmental isolate of *E. coli* from a drinking water distribution system, provided by D. Smith, South Central Connecticut Regional Water Authority, New Haven, Connecticut was used to optimize methods for fluorescent *in situ* hybridization on filter membranes for use with the Scan*RDI* following a protocol adapted from Baudart et. al. (6, 7). *Aeromonas hydrophila* obtained from the same source was used as a negative control.

Cell Growth and Preparation

E. coli and *A. hydrophila* were grown overnight at 37 ˚C on R2A agar and harvested with a sterile cotton swab into filtered autoclaved ultrapure water (FAUP). The suspension was vortexed for 30 seconds then passed through a sterile 5 µm pore-sized filter to reduce clumps of cells. The turbidity of the cell suspension was adjusted to around 15 Klett units using a Klett-Summerson Colorimeter. This represents a suspension of about 1.5 X 10^8 cells/ml. The suspension was diluted in FAUP to provide either 10^6 cells/ml for epifluorescent microscopy or 10^2 to 10^4 cells/ml for enumeration on the Scan*RDI*. The cells were filtered through 25 mm 0.4 µm pore size black polyester CB04 membrane filters (AES Chemunex 200-C2010-01).

Membranes were treated in duplicate. For epifluorescent microscopy, 1 ml of the 10^6 cells/ml suspension was filtered through polyester membranes placed over a support membrane (GE Osmonics E04WP02500) on a glass filter support with a filter chimney. For enumeration with the Scan*RDI*, the membrane was placed directly on a filter support and 100 µl of the suspension drawn through the membrane.

Prefixation Incubation

If a prefixation incubation step was done to increase the numbers of ribosomes available for hybridization, membranes were placed on a 25 mm absorbent pad (Millipore AP1002500) containing 0.65 µl of R2A broth with either chloramphenicol (Acros 227920250) at a final concentration of 100 µg/ml or nalidixic acid (Sigma N8878) at a final concentration of 10 µg/ml or a mixture of chloramphenicol and nalidixic acid. This prefixation incubation was done at 37˚C for 0.5 to 4 hours.

FISH Probes

Oligonucleotide probes analogous to the variable portion of 16S ribosomes for *E. coli* were found in relevant literature. The initial experiments were done with either the eubacterial DNA probe EUB 338, (GCTGCCTCCCGTAGGAGT) (20), a eubacterial peptide nucleic acid (PNA) probe Unibact1, (CTGCCTCCCGTAGGA) (21) or a DNA probe targeted for, but not specific for, *E. coli*, ECO 541 (CCGATTAACGCTTGCACC) that was reported by Fuchs, *et. al.* (12) to provide a bright fluorescent signal. The oligonucleotide, colinsitu, (GAGACTCAAGATTGCCAGTATCAG) (11) labeled with HRP (COL-HRP), was used as an *E. coli* specific probe with the unlabeled helper probes, Hcolin_L (ATGCAGTTCCCAGGTTGAG) and Hcolin_R, (ACCTGGAATTCTACCCCCCTCTAC) (7, 19) added to the hybridization buffer. These probes were obtained commercially as EUB 388 and ECO 541 labeled directly with Alexa 488 (EUB-Alexa and ECO-Alexa respectively) or with fluorescein (EUB-FITC and ECO-FITC) (Integrated DNA Technology). The peptide nucleic acid (PNA) probe, Unibact1was labeled with FITC (PNA-FITC) (Panagene). EUB 388, ECO 541 and Colinsitu were obtained conjugated with horse radish peroxidase (EUB-HRP, EUB-HRP, Colinsitu-HRP respectively) (Thermo Scientific and biomers.net). These 5 latter probes were obtained for use with a fluorescein tyramide signal amplification system (FITC-TSA) (Perkin Elmer) or Alexa 488-TSA (Life Technologies) described below. A noneubacterial probe, (ACTCCTACGGGAGGCAGC) (22), conjugated to HRP (NonEUB-HRP) was used as a negative control.

Fixation and Permeabilization

For fixation and permeabilization, membranes containing the cells to be hybridized with DNA probes were placed on an absorbent media pad containing 650 µl of 4% paraformaldehyde (Sigma P1648 or Electron Microscopy Sciences 15710) for 1 hour at room temperature. The membranes were transferred to a series of ethanol soaked pads (50%, 80% and 94%) for 4 minutes each at room temperature followed by a rinse by placing the membrane directly on 60 µl TE buffer (100 mM Tris HCL, 50 mM EDTA pH 8.2) for 5 minutes. After the TE rinse, membranes were placed directly placed on a solution of lysozyme from egg white (Fisher BP535-1) in TE buffer (final concentration of 5000 units /ml) at room temperature. Exposure times were varied to determine optimal time between 5 and 10 minutes. To end lysozyme activity, membranes were placed directly on 60 µl TE buffer for 5 minutes. Later, a 10 to 20 minute room temperature lysozyme inhibition step was added prior to the TE rinse by placing membranes directly on 60 µl of 0.02N HCl. This was done to inactivate the lysozyme and also eliminate any endogenous peroxidases (23, 24). The HCl exposure was followed by a final TE rinse.

A second fixation method was developed for use with PNA probes on filter membranes that had been found by other researchers to be adequate for fixation and permeabilization of both Gram negative and Gram positive cells in suspension (21) . This procedure exposed cells to 4% paraformaldehyde for 1 hour at room temperature followed by 50% ethanol at -20°C for at least 30 minutes before hybridization.

A protocol exposing oocysts to hot ethanol was recommended as an improved method to increase permeability in *Cryptosporidium.* As the goal was to detect both *E. coli* and *Cryptosporidium* on the same membrane, a third technique was examined. For

this method, membranes containing cells were placed on 50% ethanol heated to 80˚C for 10 minutes (25).

Fluorescent *in situ* Hybridization

After fixation and permeabilization, membranes with *E. coli* were placed in sterile plastic Petri dishes (35 X 10 mm) directly on a 100 µl drop of the hybridization buffer and incubated at either 46˚C or 48˚C for 2 hours in hybridization chambers. These chambers were made by placing 4.25 X 4.25 X 2" squares of florist's foam soaked in water to saturation in 4.5 X 4.5 X 2.5" tightly sealable plastic containers (Snapware). To provide adequate heat exchange, but minimize possibility of contamination of the membrane, Petri dishes used for hybridization and the following wash step were set on the florist's foam so that only the bottom of the Petri dish was in contact with water. The sealed container was placed in a water bath at the hybridization temperature. This method allowed maintenance of adequate humidity to prevent drying and proper temperature throughout the hybridization steps, but minimized the amount of hybridization buffer and thus oligonucleotide probe needed. Hybridization buffer was composed of 0.9 M NaCl, 20 mM Tris HCl pH7.2, 0.01% sodium dodecyl sulfate (SDS) and 20 % formamide in sterile ultrapure water, except the PNA probe which was hybridized with 100 mM NaCl, 20 mM Tris-HCl pH 9, and 0.5% SDS. Formamide was omitted in some cases when the ECO541 or Colinsitu-HRP probe was used (12). Addition of dextran sulfate (DS) in the hybridization buffer (100 mg/ml) was compared to hybridization buffer without DS. Probe concentrations were varied to determine the optimal concentration for adequate fluorescent intensity for detection with the Scan*RDI*. Hybridization times ranged from 1

to 2.5 hours. After hybridization, membranes were placed in prewarmed Petri dishes on 100 µl prewarmed wash buffer (180 mM NaCl, 20 mM Tris-HCl pH 7.2, 5 mM EDTA and 0.01 % SDS) for 30 minutes at 48˚C. After the wash step, all membranes were rinsed by placing membranes on 0.65 µl TNT buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20) for 5 minutes.

Tyramide Signal Amplification

If cells were hybridized with probes conjugated with HRP additional steps were done for the tyramide signal amplification technique according to the manufacturer's specifications for Fluorescein-TSA (TSA-FITC) (Perkin Elmer) or Alexa 488-TSA (Life Technologies). In short, for cells with probes conjugated with HRP, membranes were placed on tyramide-fluorescein or tyramide-Alexa 488 (diluted 1:50 or 1:100 respectively) for 10 minutes followed by rinsing in TNT as described above.

Imaging and Enumeration

Membranes filters were placed on absorbent pads until dry then examined immediately using the Zeiss Axioskop epifluorescent microscope or the Scan*RDI*.

For epifluorescent microscopy, dry membranes were mounted on slides using Vectashield mounting medium without DAPI (H-1000 Vector Lab) and examined with an Axioskop 50 (Zeiss) epifluorescent microscope with a 100X oil objective using a filter set appropriate for FITC (Ex/Em 460-500nm/515-550 Chroma 41001). Cells enumerated by epifluorescent microscopy were imaged with an AxioCam 412 using Axiovision software (Zeiss) at either 100 or 150 msecs and the images analyzed with Metamorph software (MetaImaging Systems ver. 7.7) to quantitatively determine the fluorescent

intensity of the probed cells. The fluorescent intensities that had been generated by the different methods were compared to ascertain improvements in signal intensity resulting from the changes in methodology.

Membranes for enumeration using the Scan*RDI* were placed on a 25 mm cellulose support membrane (GE Osmonics E04WP02500) containing 100 µl of mounting medium on the specially designed Scan*RDI* holder. Initially TNT buffer and later DABCO/glycerol mounting medium made according to the US EPA methods for detection of *Cryptosporidium* (26) were used for the mounting medium. The advantage of using glycerol based mounting medium over the water based TNT buffer was the prevention of drying of the membrane on the holder, allowing a longer microscopic examination of the membranes. The AES Chemunex support membranes (AES Chemunex 200-C2107-01) were not used as they are degraded by glycerol.

The holder was then placed into the scanner and the membrane surface was scanned for fluorescent events using a 488nm laser. For initial examination of all membranes, the events detected were analyzed by the software using the application designated by Chemunex as total viable count (tvc). After scanning, the holder was placed on the automated stage of an epifluorescent microscope (Nikon Optiphot) using the B2A filter set appropriate for FITC detection, where the Scan*RDI* software directed the stage to each event on the data screen. The membranes were analyzed in the 'validate all' mode, allowing visualization of all fluorescent particles above a threshold intensity.

When using epifluorescent microscopy, in addition to quantification of fluorescent intensity, the number of cells labeled with FISH probes were compared to cell numbers on membranes stained with 100X SYBR Green (SG) for 10 minutes at room

temperature. SG is a nucleic acid stain and should label all cells present on a membrane. SG has also been used previously for total enumeration of cells by the Scan*RDI* (4). ChemChrome V6, a proprietary reagent developed by AES Chemunex, is reduced by esterase activity to release free fluorescein inside viable cells. Chemchrome V6 was used to enumerate cells by the Scan*RDI* according to manufacturer's instructions. Briefly, after filtering cells through the polyester CB04 membrane, the membrane was placed on a pad containing 600 µl of Chemsol A and incubated at 37˚C for 1 hour. The membrane was then moved to a pad with 600µl of ChemChrome diluted 1:100 in Chemsol B and incubated at 30˚C for 30 to 90 minute. The membrane was then read immediately using the tvc application on the Scan*RDI.*

Plate counts were done in duplicate using the drop plate method (27) on R2A agar incubated at 36˚C overnight.

Development of a FISH Application for Use with the Scan*RDI*

Several steps are necessary to adjust the Scan*RDI* application for different labeling protocols. The initial determination of whether a fluorescent event is a targeted organism or an artifact is the threshold of the fluorescent intensity. This can be adjusted, but when the fluorescent intensity of the target organism is low, lowering the threshold will allow interference from background or low intensity artifacts which may confound the detection of labeled organisms. For the targets examined here, no change in threshold was required. The fluorescent events are then screened by several characteristics of fluorescence, such as the fluorescent intensity, color, and signal shape. Those particles that express the same characteristics or discrimination factors as properly labeled cells are

shown on the ‘results’ screen. The membranes observed in the ‘validate all’ mode allow examination of all fluorescent events above the intensity threshold. By examining the characteristics of false negative and false positive particles, a determination can then be made as to which, if any, of the discrimination factors need be adjusted in order to increase the number of true positive cells that appear in the results screen and reduce the number of extraneous artifacts. Development of a FISH specific application was done by sorting each of discrimination factors for all FISH positive target organisms. This provided the range of each of those 10 discrimination factors manifest by those organisms labeled with FISH recorded in the discrimination table. For the FISH application, the half width was also increased after a comparison of the positive targets from data and results screens showed that the discrepancy between the targets not shown on the results screen but determined to be positive on the data screen, was due to a half width factor that was too low. The half width is not shown in the discrimination table with the other discrimination factors on the Scan*RDI* software, but is found in a table associated with each individual particle. All the ranges were entered into a new application designated FISH. All membranes examined during the optimization procedures were done using the FISH application in the ‘validate all’ mode to verify that all cells labeled by with the FISH probe could be viewed on the results screen of the Scan*RDI*.

Results

Prefixation Incubation with Chloramphenicol

Addition of an incubation period with low nutrient broth containing antibiotics prior to hybridization was recommended to increase ribosome content and fluorescent intensity and thus increase the numbers of cells detected by FISH without increasing the number of cells on the membrane filter (6, 28). At the onset, use of chloramphenicol was explored to increase the fluorescent signal. As shown in Figure 2.1, the number of cells enumerated after 30 minutes incubation with chloramphenicol then labeled with the ECO-Alexa probe were equivalent to the cells detected by SG labeling. The image in Figure 2.2 highlights the increase in fluorescence after 30 minute incubation with R2A containing chloramphenicol compared to hybridization without an preincubation step. No Metamorph data analysis was done at this time, so quantification of fluorescent intensity was not available.

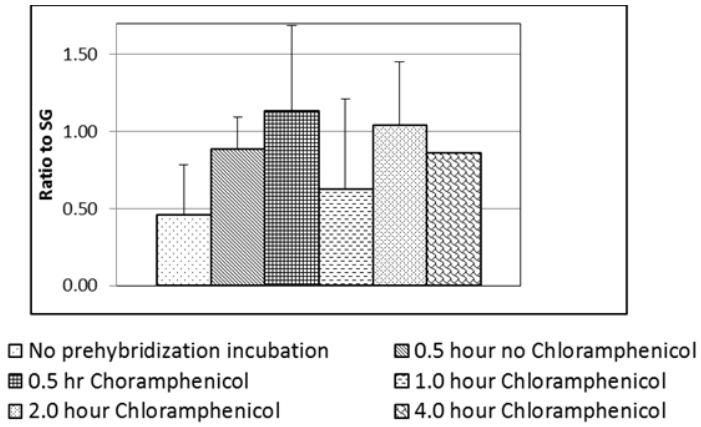

Figure 2.1. Comparison of Prefixation Times using Chloramphenicol
Ratio of numbers of E. coli cells probed with ECO-Alexa by FISH techniques to number of cells stained with SG n=3.

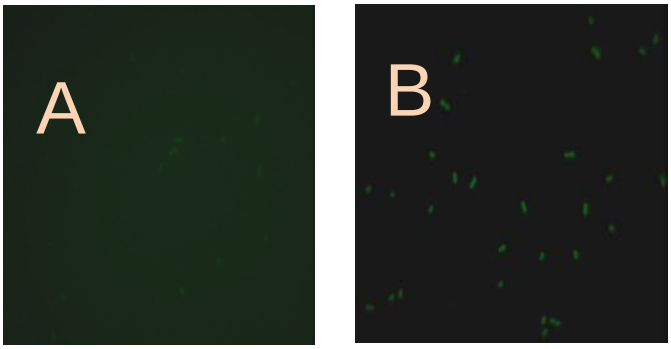

Figure 2.2. Images of E. coli Cells Probed with ECO-Alexa
A) without prefixation incubation and B) with 30 minute prefixation incubation with chloramphenicol in R2A broth. (Both images taken with 150 msec exposure time)

Comparison of Probes

Fluorescent *in situ* hybridization using eubacterial DNA probes, EUB-FITC and EUB- Alexa, and the eubacterial PNA-FITC probe (all at 5 ng/µl final concentration) was compared to results obtained from staining with SG by enumerating cells with the epifluorescent microscope and by quantifying fluorescent intensity after imaging. Cell numbers detected by the Scan*RDI* were compared to those obtained by plate counts. As seen in Table 2.1, cell numbers as determined by SG show that nearly all cells on the membrane were probed by the FISH methods using EUB 338 and Uni1 PNA probe after a 30 minute prefixation incubation. Fluorescent intensity was greater with the PNA probe and PNA methodology than for either of the DNA probes (Figure 2.3). Not indicated by the comparison of the intensity is the presence of large numbers of brightly stained artifacts found when using the PNA probe which would have made enumeration with Scan*RDI* extremely difficult (Figure 2.4). The EUB probe labeled with Alexa 488 was slightly more intense than when the probe was labeled with FITC. Although the number of cells probed was equivalent to the number of cells labeled with SG, the fluorescent

intensity of FISH labeled cells was not adequate for detection by the Scan*RDI* without decreasing the threshold to the point that faint background particles would interfere with enumeration of the FISH labeled cells. The number of cells probed with ECO-Alexa with the highest tested probe and detected by the Scan*RDI* concentration was only 8% (Table 2.2).

Table 2.1. Comparison of Cell Numbers Determined by Different FISH Probes to Plate Counts and SYBR Green. Number of *E. coli* as determined by epifluorescent microscope counts using SG, EUB-FITC, EUB-Alexa and the PNA-FITC probe with FITC n=3.

Enumeration Method	Log of Mean	STDEV
SYBR Green	6.16	0.04
EUB Alexa	6.17	0.04
EUB FITC	6.02	0.10
PNA FITC	6.01	0.15
R2A Plate Count	6.19	0.07

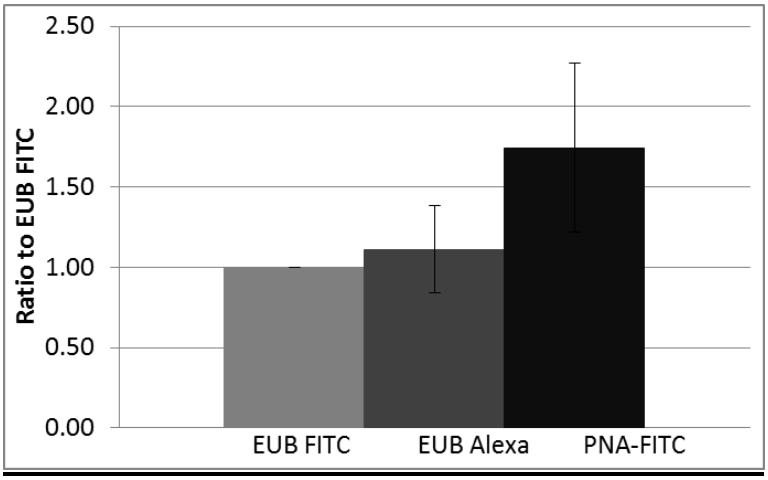

Figure 2.3. Comparison of EUB-FITC, EUB-Alexa and PNA-FITC Probe Intensity Ratio of fluorescent intensity of cells probes with EUB-FITC to EUB-Alexa and PNA-FITC as determined by Metamorph analysis. The fluorescent intensity of cells labeled with SG, not shown on this graph, were 52.9 times that of EUB-FITC n=3.

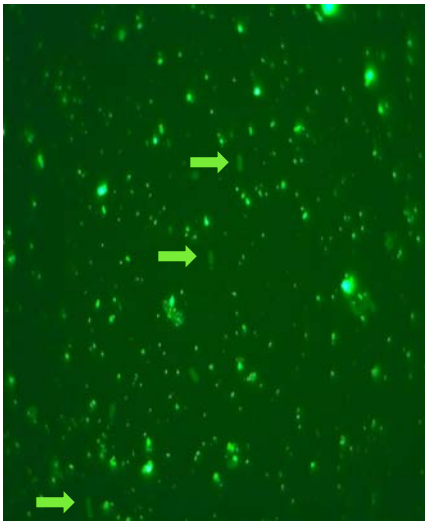

Figure 2.4 Artifacts Present after Hybridization with PNA-FITC
Cells are indicated with an arrow.

Table 2.2. Optimization of ECO-Alexa Probe Concentration. Cells labeled with ECO-Alexa probe with different concentrations of probe in hybridization buffer enumerated by Scan*RDI* compared to cell number as determined by plate counts. 5 ng/µl is 0.8 pmol/µl final concentration n=1.

	# Cells	Ratio Scan*RDI* counts to Plate Count
Negative control	1.00	0.00
5 ng/µl probe	71.00	0.08
2.5 ng/µl probe	3.00	0.00
1.25 ng/µl probe	17.00	0.02
Plate counts	876.67	1.00

Use of SYBR Green for
Detection of Cells with Scan*RDI*

Although SG has been used in the past by this laboratory to enumerate total cells with the Scan*RDI* (4), problems occurred when attempting to compare numbers of FISH probed cells with SG stained cells due to the presence of between 1500 and 6000 SG stained artifacts per membrane. This confounded attempts to verify cell counts. Numerous procedures were tried to eliminate these particles with little success. It was

decided that either plate counts or ChemChrome V6 would be used to determine the viable cell number to compare with the number of cells found by FISH when the Scan*RDI* was used to enumerate *E. coli*. When cells were read with epifluorescent microscope, SG counts were used for comparison.

Increasing Permeability of *E. coli* Cells

Cells hybridized after overnight treatment at -20˚C on 50% ethanol followed by 80% and 94% ethanol treatment at room temperature showed no increased fluorescent intensity compared to cells that were hybridized after 4 minute treatment on 50%, 80% and 94% ethanol. Treatment with hot ethanol (80˚C) for 10 minutes followed by hybridization rendered cells so faintly labeled that they were difficult to enumerate and the hot ethanol treatment was not pursued as a permeabilization method for *E. coli*.

Incubating membranes containing cells for 10 minutes at room temperature with lysozyme prior to hybridization with EUB-FITC increased the fluorescent intensity as measured by Metamorph by 20%, but the average intensity was still only 3% as bright as that produced by staining with SG.

Due to these preliminary findings, it was determined that although the FISH method with directly labeled probes hybridized of nearly all cells on the membrane, additional techniques would be needed to be employed in order to provide adequate fluorescent intensity for detection of single cells by the Scan*RDI*.

Tyramide Signal Amplification

Although the manufacturer does not recommended use of the TSA reagents with membrane filters, initial experiments found that placing polyester membranes containing *E. coli* hybridized with EUB-HRP directly on diluted tyramide fluorescein reagents in the dark for 10 minutes, produced an enhanced fluorescent signal, with the average intensity between 35 and 75% of that of SG. Directly labeled cells had a fluorescent intensity of between 2 and 6% when compared to SG. Because of these findings, all remaining optimization were done using TSA.

Optimization of Protocol
for EUB-HRP and ECO-HRP

Results using the data obtained from Metamorph with *E. coli* probed with ECO-HRP showed that there was no improvement in the minimum fluorescent intensity by use of nalidixic acid, combining nalidixic acid and chloramphenicol or by increasing incubation time to 60 minutes above that of cells incubated with chloramphenicol for 30 minutes (Table 2.3). In order to minimize the time required for the FISH procedure, initial experiments analyzed with the epifluorescent microscope were done after prefixation incubation on R2A with chloramphenicol for 30 minutes. It was found, however, that the cell size and fluorescence manifested after 30 minutes incubation with chloramphenicol was often not adequate for cells to be detected by the Scan*RDI* without lowering the threshold intensity. When the threshold is lowered, more artifacts are detected by the scanner, increasing the time required for examination of membranes. Although the minimum fluorescent intensity was no brighter, by using nalidixic acid and

increasing the incubation time to 60 minutes, the cell size was increased, which allowed detection of *E. coli* cells by the Scan*RDI* without lowering the fluorescent threshold.

Table 2.3. Comparison of Antibiotics for Prefixation Incubation
Comparison of the minimum fluorescent intensity from images analyzed with Metamorph after prefixation incubation with chloramphenicol and/or nalidixic acid compared to chloramphenicol after 30 minute incubation n=5.

Minimum Fluorescent Intensity	Mean	StDev
Chloramphenicol 30 minutes (C30)	1.00	0.00
Chloramphenicol 60 minutes	1.09	0.17
Nalidixic Acid 30 minutes	1.06	0.25
Nalidixic Acid 60 minutes	1.41	0.47
Chloramphenicol and Nalidixic Acid 30 minutes	1.03	0.03
Chloramphenicol and Nalidixic Acid 60 minutes	0.98	0.05

Determination of the optimal probe concentration using ECO-HRP was done on 2 days using the images taken through the epifluorescent microscope and analyzed using Metamorph software. Shown in Figure 2.5, the optimal concentration was determined to be 0.5 pmol/µl, with no advantage to increasing the concentration.

Maximum peak intensity determined by the Scan*RDI* was brighter when amplified with TSA-Alexa, but minimum intensity was not. Because the range for peak intensity in the FISH application is more dependent on the minimum peak intensity, there was no improvement in detection by the Scan*RDI*. As adding dextran sulfate to the hybridization buffer or using the TSA-Alexa 488 tyramide system did not increase detection of the number of cells by the Scan*RDI*, these methods were not pursued. (See Figure 2.6).

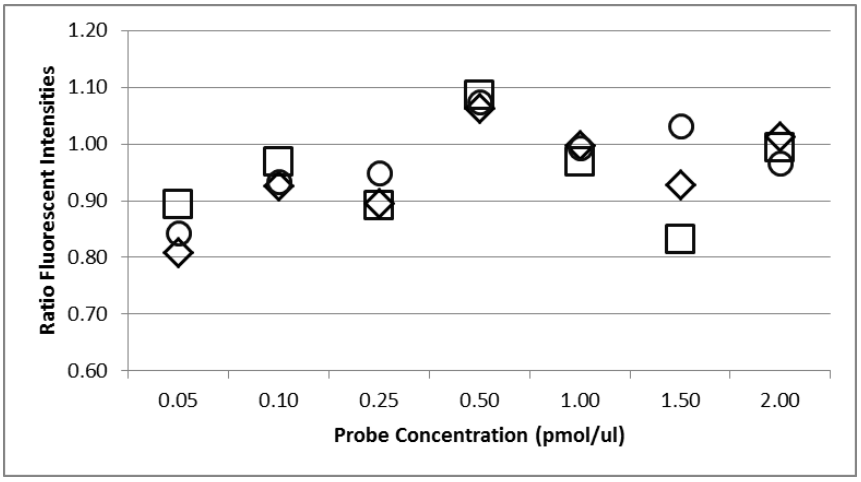

Figure 2.5. Optimization of ECO-HRP Probe Concentration
Comparison of the fluorescent intensity normalized to the fluorescent intensity of 0.5 pmol/µl undiluted probe (minimum (squares), mean (diamonds) and maximum (circles)) Concentrations over the range of 0.05 to 2.0 pmol/µl of probe final concentration in hybridization buffer n=2.

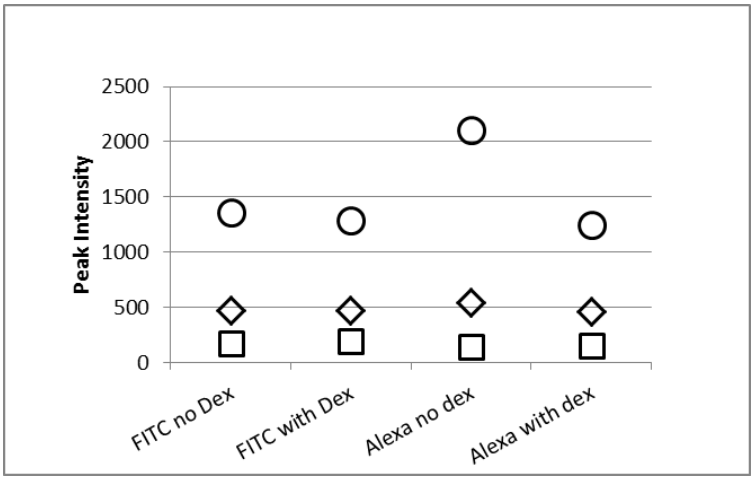

Figure 2.6. Use of Dextran Sulfate and Alexa 488-TSA
Minimum (squares), mean (diamonds) and maximum (circles) peak intensities of *E. coli* cells determined by the Scan*RDI* after prefixation incubation with NA for 1 hour and FISH probing with ECO-HRP with or without DS followed by either TSA-FITC or TSA-Alexa amplification n=2.

The detection of cells by FISH compared to viable cell counts as determined by Chemchrome was between 32 and 75% when coupled with TSA-FITC. This is an improvement over initial detection levels of 8% (shown in Table 2.2).

Analysis using Metamorph intensity data determined that there was no difference in fluorescent intensity after hybridization for 1 hour compared to 2 hours, preheating the hybridization buffer and Petri plates improved intensity only slightly. Changes in fluorescent intensity due changes in hybridization times is shown in Figure 2.7.

Verification of the FISH
Application After TSA

It was determined that over 96 % of the fluorescent events deemed positive for the target on the data screen were determined by the Scan*RDI* software to be targets on the results screen. This indicated that the newly developed application was valid for use in the results mode when using FISH probes in conjunction with TSA-Fluorescein.

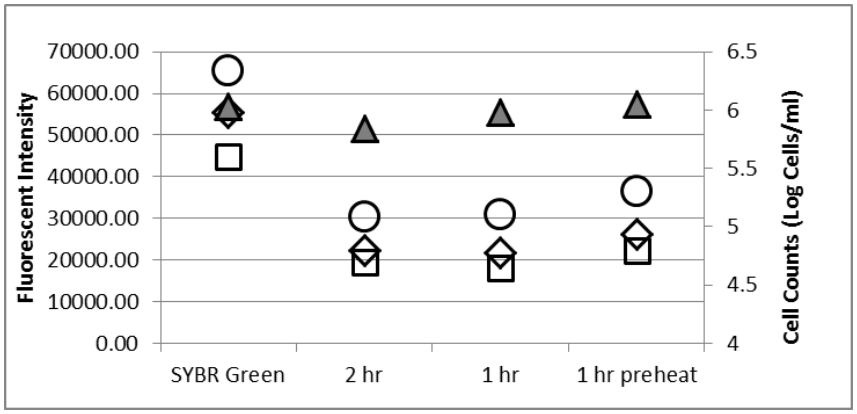

Figure 2.7. Optimization of Hybridization Time for ECO-HRP
Minimum (squares), mean (diamonds) and maximum (circles) peak intensities and log of the counts of *E. coli* cells (triangles). Intensities were determined by Metamorph after imaging for 100 msec exposure. Hybridization with ECO-HRP for 2 hours, 1 hour and 1 hour after preheating buffer and plates to 48˚C followed by TSA-FITC amplification n=2.

Table 2.4. Frequency Thresholding and Discriminant Settings used for Detection of FISH Labeled Cells and Oocysts with the Scan*RDI*.

Frequency Table Thresholding

Threshold	
K_o	0
K_1	0.05
Pretrigger	0
Trig Delay	5

Discriminants	Min	Max
Lines	2	20
Samples	7	50
S/P	0.16	1.2
Peak Value	99	6000
T/P	0.09	1.2
SI (AS)	2	X
SI (HW)	8	X
2D Gaussian	X	2500
Half Width	X	18
Peaks	X	3
Wiggles	X	10

Detection of *E. coli* with
Colinsitu and Helper Probes

A four hour prefixation incubation with nalidixic acid followed by probing with COL-HRP in conjunction with unlabeled helper probes, followed by the TSA reaction in a modification of the protocol provided by Baudart et. al. (7), allowed consistent detection with the Scan*RDI*. The Scan*RDI* using the FISH application identified between 70 and 90% of the cells enumerated by plate counts (Table 2.5). To later be able to perform FISH with *Cryptosporidium* on the same filter, the hybridization buffer was made without formamide and the membranes were incubated at 48˚C (see following chapters).

Table 2.5. Comparison of Scan*RDI* Detection of *E. coli* Hybridized with FISH to Plate Counts. *E. coli* were hybridized after 2 or 4 hour prefixation incubation with Colinsitu-HRP and helper probes followed by TSA

Date	11/20/12	12/3/12	12/6/12	12/10/12	12/17/12	12/19/12
2 hr. w/ formamide	0.68	1.12	0.94			
4 hr. w/ formamide	0.63	1.01	0.99			
4 hr. w/o formamide		0.84	0.88	0.9	0.71	0.7

.

Discussion

Three elements were key to optimizing the FISH procedure to allow successful detection of FISH labeled *E. coli* on membrane filters by the Scan*RDI.* A prefixation incubation of 2 to 4 hours on nalidixic acid was essential to providing sufficient numbers of ribosome. The second important step was the lysozyme permeabilization. This allowed entry of the probe with the large horse radish peroxidase label into the cell, but maintained the cell shape. Thirdly, the use of TSA provided the strong fluorescent signal with little background fluorescence that the Scan*RDI* required.

None of the probes directly labeled with FITC or Alexa 488 produced adequate signal for detection of more than 10% of *E. coli* cells by the Scan*RDI*. Peptide nucleic acid (PNA) probes have the advantage of being uncharged and so enter the cells more easily than DNA oligonucleotide probes (9, 21), however the PNA probe obtained from Panagene added so many artifacts to the filter that it precluded its use with the Scan*RDI*. Labeling of the probe with Alexa 488 increased fluorescence only slightly over the same probe labeled with FITC.

Increasing the numbers of ribosomes by prefixation incubation of the cells on R2A with antibiotics provided one of the biggest gains in fluorescence. There was little

difference in intensity as determined by the Metamorph software by using chloramphenicol in the medium compared to nalidixic acid, but as chloramphenicol inhibits bacterial growth by inhibiting protein synthesis (29), the cells remained small with increased fluorescence. The mode of action of nalidixic acid is inhibition of DNA gyrase (30, 31), allowing an increase in ribosomes but interfering with cell division and so that the bacteria continue to increase in size. The larger cells produced by incubation with nalidixic acid are more easily detected by the Scan*RDI*. Although 2 hour incubation with nalidixic acid was adequate when using laboratory grown cells, cells from the environment are likely to require a longer incubation time to produce ribosomes in numbers required for the fluorescent intensity needed by the Scan*RDI* .

Fixation and permeabilization with 4% paraformaldehyde and the series of ethanol baths (6) was adequate for *E. coli* when using directly labeled probes such as ECO-FITC or EUB-Alexa, but incubation with lysozyme for 10 minutes increased permeabilization by breaking down the peptidoglycan in the cell wall which allowed entry of the probe-HRP into the cell and proved important later when the protocol was used for *Cryptosporidium* (see following the chapters). Adding the HCl step to inactivate the lysozyme lowered the incidence of apparent leaking of fluorescein out of the cells seen during microscopic examination of the filter membranes. The HCl step had the additional benefit of eliminating possible endogenous peroxidase, important for use with environmental samples and because the ultimate goal was using the technique to label other organisms, which may contain peroxidases, on the same filter at the same time. The ideal concentration of probe was 0.5 pmol/µl, a balance between increasing fluorescence and reducing the use of the expensive HRP labeled probe.

Hybridization time was kept between 1.5 and 2.5 hours. There was no difference in results over this time span. Prewarming the buffer and the Petri plates used for the hybridization also allowed hybridization for 1 hour, but decreased the number of cells hybridized if incubation time was extended due to drying of the buffer.

The other key element in the success of the procedure was the use of tyramide signal amplification, also called catalyzed reporter deposition (CARD-FISH). The TSA technique uses oligonucleotide probes directly coupled with horse radish peroxidase (32, 33) or a probe that can be coupled later with HRP, e.g. biotin which binds to avidin-HRP (34), or fluorescein which can interact with an α-fluorescein-HRP antibody (35). HRP on the hybridized oligonucleotide converts tyramide labeled with a fluorophore into an activated intermediate that binds to adjacent proteins. Because this is an enzymatic reaction, multiple deposition of the activated tyramide occurs in a short time. producing a large amplification of the fluorescent signal (6, 32, 34, 36).

By further adapting the protocol developed by Baudart et. al. (6, 7), specifically using TSA with Colinsitu-HRP plus unlabeled helper probes (7, 11, 19), and prefixation incubation with nalidixic acid for two to four hours enabled detection by the Scan*RDI* of a high percent of *E. coli* cells compared to enumeration by plate counts (70 to 112%). Higher percentages were obtained when formamide was in the hybridization buffer.

The FISH protocol (Table 2.6) including incubation with nalidixic acid, fixation with 4% paraformaldehyde, followed by a series of ethanol dehydration steps and lysozyme permeabilization increased the opportunity for the probe-HRP to enter the *E. coli* cell. That procedure, coupled with TSA provided a bright signal that enabled consistent detection of *E. coli* probed cells by the Scan*RDI* in numbers matching that of

enumeration by plate count methods. When using the COL-HRP probe system this allowed identification and enumeration of small numbers of *E. coli* from suspensions within one day.

Table 2.6. Flow chart of FISH Hybridization Protocol. Membranes are placed on the solutions indicated, either directly or on an absorbent pad.

FISH Hybridization
R2A broth with nalidixic acid 1 – 4 hours ↓ 4% paraformaldehyde 1 hour ↓ 50 % 80 94% ETOH bath 4 min each ↓ 60 µl TE rinse 5 min ↓ Lysozyme 10 min ↓ 60 µl 0.02 N HCL 10 min ↓ 60 µl TE rinse 5 min ↓ Hybridize 90 min at 48 °C ↓ Wash buffer 100 µl 30 min 48°C ↓ Rinse TNT buffer 5 min ↓ FITC tyramide 75 µl 10 min ↓ Rinse TNT buffer 5 min ↓ Dry on cellulose pad until analyzed

FLUORESCENT *IN SITU* HYBRIDIZATION OF *CRYPTOSPORIDIUM*

Introduction

Organisms in the genus *Cryptosporidium* are monoxenous, intracellular protozoan parasites requiring a host for both growth and reproduction. *Cryptosporidium spp*. have a complex lifecycle consisting of both sexual and asexual reproduction. The infective stage is an oocyst, usually containing four sporozoites, that is passed from the host in the feces (37, 38). The means of transmission is by fecal-oral route, often through water or in food irrigated or washed with contaminated water (39).

Critical to the survival of *Cryptosporidium* in the environment is the structure of a unique, thick oocyst wall (~40 nm thick) (40) which protects the infective sporozoites (41). This wall structure has three distinct layers: the outer layer of glycoprotein, a central glycolipid/lipoprotein that gives rigidity and elasticity to the oocyst and a thick filamentous inner glycoprotein layer (40).

Cryptosporidium normally inhabit the gastrointestinal tract of their host (37). Although infection can be asymptomatic, the hallmark of the cryptosporidiosis is frequent, watery diarrhea which is caused by direct epithelial injury and by inflammation in response to the presence of the parasites. Diarrhea can be accompanied by abdominal cramping, nausea, vomiting, fever, and weight loss. The illness is normally self-limiting in immunocompetent individuals, but people with defects in immunity can have a more severe and longer lasting diarrhea. In patients with HIV/AIDS, the prolonged diarrhea can lead to wasting and death (37, 38, 42-44).

Disease progression, as manifest by severity of clinical symptoms, is influenced by the concentration of viable organisms ingested, the host immune system and the strain of the parasite. The infective dose in healthy individuals has been reported to be from 10 to 132 oocysts, dependent on the strain (42, 44), although it has been extrapolated from outbreaks that certain people could develop the disease following ingestion of only one oocyst (37).

Cryptosporidium is transmitted by the fecal-oral route and results from ingestion of oocysts from contaminated water or food as well as direct contact with infected people or animals. Because of the ease with which oocysts enter the watershed and because they have a high resistance to chlorination, water is considered the main method of transmission (45). In view of the large numbers of oocysts shed by infected animals and the low infectious dose needed to cause disease even in healthy populations, monitoring water for *Cryptosporidium* oocysts seems the most likely means to have the largest effect on the public health.

Detection of *Cryptosporidium* from water using the EPA method 1622/1623 relies on labeled antibodies to concentrate and identify *Cryptosporidium* (26, 46). Samples containing oocysts are placed on a slide and exposed to an anti-*Cryptosporidium* antibody and diamidino phenylindole dihydrochloride (DAPI). The entire well containing the oocysts is then examined with epifluorescent microscopy and differential interference contrast microscopy. This is a laborious and time consuming method, with the possibility of false negatives when oocysts are removed from slides by washing steps and false positives, since the antibodies cross react with material in environmental samples.

Fluorescent *in situ* hybridization (FISH) is a technique used to identify whole cells by introducing into the cell synthetic DNA oligonucleotide probes labeled with a fluorescent dye. The probes are complimentary to target regions of the ribosomal RNA and can be designed to identify different taxonomic levels from universal eukaryotic, euarcheal or eubacterial probes to species specific probes. Because organisms contain multiple copies of rRNA, there is a natural amplification of the target sequence enabling visualization of the cell after FISH using epifluorescent microscopy (47, 48). Unlike antibody labeling, properly designed FISH probes don't cross react with extraneous material or with related but untargeted species. Because RNA is not long lived, use of FISH in determination of viability in *Cryptosporidium* has been reported (49, 50). The use of FISH therefore, provides both a species specific identification and viability assessment (51).

The major impediment to use of FISH with *Cryptosporidium* is the complex thick wall surrounding the oocyst, which must be permeabilized to allow the penetration of the fluorescently labeled oligonucleotide probe, especially when coupled with the large horse radish peroxidase molecule used in tyramide amplification methods (see below).

FISH can be done either in suspension (25, 49, 50, 52), after filtering the oocysts through polycarbonate or polyester membranes (51, 53), or on a glass slide.

After permeabilization of the oocyst, hybridization proceeds using a hybridization buffer containing the fluorescently labeled probe at 46 to 48°C for one to two hours. Technologies exist to amplify a signal using an enzyme-based system called catalyzed reporter deposition (CARD) or tyramide signal amplification (TSA). In this procedure, the probe is labeled with horse radish peroxidase (HRP). After hybridization, the cell is

exposed to tyramide molecules that have been coupled to a fluorophore such as fluorescein. In the presence of HRP, the tyramide is converted a reactive intermediate which immediately covalently binds to proteins, localizing the signal near the probe. Since many tyramide conjugates are catalyzed by one HRP molecule, this reaction increases the signal up to 100 times (54). The FISH labeled oocysts can be enumerated directly with epifluorescent microscopy (49-51, 53, 55) or flow cytometry (25, 52) without TSA amplification.

This project sought to use solid phase laser cytometry (SPLC) in the Scan*RDI* to compare the number of oocysts detected with FISH to the number of oocysts identified using the EPA slide method and FAb. Before proceeding to environmental samples, the FISH protocol developed with *E. coli* was optimized for use *Cryptosporidium parvum* on the membrane filters required for detection by the SPLC. This chapter describes the optimization using control oocysts.

Methods

Organisms, Probes, and Antibodies

Cryptosporidium parvum oocysts, obtained from Sterling Parasitology Laboratory at the University of Arizona, were used to optimize methods for fluorescent *in situ* hybridization on membrane filters.

An oligonucleotide probe used to identify *Cryptosporidium parvum*, CRY-1 5′-CGGTTATCCATGTAAGTAAAG-3′ (49), was labeled directly with horse radish peroxidase (CRY-HRP) (biomers.net and Thermo Scientific) or labeled with biotin CRY-

B) (Integrated DNA Technology). Upon receipt, the probes were diluted in water to 100 pmol, aliquoted into 20 µl amounts and stored at -20˚C.

During optimization, a fluorescein labeled polyclonal goat α-*Cryptosporidium* (α-CRY) (Lifespan Biosciences) diluted in PBS was used to enumerate oocysts for comparison with FISH probed oocysts.

Fluorescent *in situ* Hybridization

For initial studies, oocysts were permeabilized following procedures described by Deere et. al. (25). Briefly, oocysts were fixed in suspension by adding 10 µl of $1X10^8$ oocysts/ml to 1 ml of paraformaldehyde (obtained from Electron Microscopy Sciences at 16% and diluted in water to 4%) and allowed to sit for 1 hour at room temperature. After centrifugation at 13,000 g (Sorvall MC12V), the supernatant was removed and the oocysts were resuspended in 50% ethanol in phosphate buffered saline (pH 8.0) (8 g NaCl; 0.2 g KCl; 1.15 g Na_2HPO_4, anhydrous; and 0.2 g KH_2PO_4 per liter of water) and heated at 80˚C for 10 minutes. The suspension was then centrifuged as above to remove the ethanol and hybridization was carried out by adding hybridization buffer containing 0.9 M NaCl, 20 mM Tris HCl, and 0.05% SDS with a final concentration of 1 pmol/µl CRY1 probe. After the two hour incubation at 48°C in a water bath, the oocysts were removed from the hybridization buffer by centrifugation, resuspended in 100 µl wash buffer (180 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS [pH 7.2]) then incubated at 48°C for 30 minutes. After centrifugation as above, the supernatant was removed and 100 µl TNT buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20) added to rinse. After centrifuging as above, 75 µl of FITC-tyramide (Perkin Elmer)

diluted 1/50 according to manufacturer's instructions was added and allowed to set at room temperature for 10 minutes in the dark. The oocysts were centrifuged again, the TSA-FITC removed and the pellet was rinsed again with TNT buffer. The oocysts were placed on a slide and, after drying, 10 µl of Vectashield ® mounting medium (Vector Laboratories) was added followed by a coverslip. Slides were visualized immediately. This method was used as a comparison for FISH methods developed for use with the black 25 mm polyester membrane filter described below.

To optimize FISH techniques for use with the Scan*RDI*, the protocols above were adapted from a procedure developed by Baudart et. al. (6) and modified in this lab using *E. coli*. When membranes were to be examined with epifluorescent microscope, oocysts were diluted in sterile water to 1 x 10^5 oocysts/ml and 1 ml was filtered through a 25 mm 0.4 µm pore-sized black CB04 polyester membrane (AES Chemunex) over a white 25 mm 0.45 µm pore-sized mixed cellulose membrane (GE Healthcare) and mounted on a glass 25 mm filter with a funnel. For the Scan*RDI*, oocysts were diluted to 1 x 10^3 oocysts/ml then 100 µl were filtered to the center of a polyester membrane giving membranes with approximately 100 oocysts. After filtration the polyester membranes were placed on absorbent media pads (Fisher AP1002500) containing 650 µl of 4% paraformaldehyde for 1 hour at room temperature.

Comparisons were made between the permeabilization method used previously with *E. coli* (see the preceding chapter) and the hot ethanol treatment described above, but adapted for use with a membrane filter.

For the hot ethanol treatment, the membranes were placed on a pad containing 50% ethanol/PBS preheated to 80˚C in a 10 X 35 mm Petri dish (BD Falcon). The Petri

dishes were placed in a humidifying chamber described in the preceding chapter, and preheated to 80˚C and the chamber placed in an 80˚C water bath for 10 minutes. After the permeabilization treatment, the oocysts were hybridized as described below.

The method for permeabilization using lysozyme developed for *E. coli*, described in preceding chapter, was tested for use with *Cryptosporidium*. The membranes with oocysts were transferred to a series of ethanol soaked pads (50%, 80% and 94%) for 4 minutes each at room temperature, rinsed by placing the membrane directly on 60 µl TE buffer (100 mM Tris HCL, 50 mM EDTA pH 8.2) for 5 minutes. After the TE rinse, membranes were placed directly placed on a solution of lysozyme (Fisher Cat # BP 535-1) in TE buffer (final concentration of 5000 units /ml) at room temperature for 10 minutes. To end lysozyme activity, membranes were placed directly on 60 µl of 0.02N HCl for 10 to 20 minutes. This was done to inactivate the lysozyme and also eliminate any endogenous peroxidases (23, 24). The HCl exposure was followed by a final TE rinse. The oocysts were then ready for hybridization.

Membranes were placed on 100 µl of the hybridization buffer described above, in 10 X 35 mm Petri dishes. The Petri plates were set in a humidity chamber as described in the previous chapter and the entire sealed container was placed in a water bath at 48˚C for 1.5 to 2.5 hours. The concentration of the probe in the hybridization buffer was varied between 0.1 and 2 pmol/µl to determine the optimal concentration. Hybridization at 46˚C with addition of 20% formamide was compared to conditions described by Deere (25) above, with incubation at 48˚C with no formamide in the buffer. Buffer with 20% formamide was tested in order to determine if *E. coli* and *Cryptosporidium* could both be

detected using the hybridization buffer used previously to probe *E. coli* (see the preceding chapter). Buffers were tested with varying concentrations of formamide from 0 to 20%.

After the hybridization period, the membranes were moved onto prewarmed wash buffer for 30 minutes at 48˚C. Membranes were removed from wash buffer and placed on 60µl TNT buffer to rinse before reaction with tyramide-fluorescein, diluted 1:50, for 10 minutes followed by a final rinse in TNT as described in previous chapter for *E. coli*. When CRY-B was used, filters were incubated on streptavidin-HRP before transferring them onto TSA-FITC. The membranes were tested for optimal storage conditions after hybridization by holding at 4˚C on TNT buffer or dried and placed at 4˚C for 3 to 7 days and the fluorescent intensity of the oocysts compared.

Other protocols were compared to this standard including addition of dextran sulfate and Tween 20 (see the preceding chapter).

Membranes examined either by epifluorescent microscopy mounted on slides with Vectashield ® mounting medium without 4',6-diamidino-2-phenylindole (DAPI) (H-1000 Vector Lab) or with the Scan*RDI* after mounting in DABCO/glycerol mounting medium (2 g of DABCO (Sigma Chemical Co.) in 100 mL of warm glycerol/PBS (60% glycerol, 40% PBS) (46).

Fluorescent Antibody Labeling

Oocysts were identified with α-*Cryptosporidium* antibodies labeled with α-FITC as described in EPA Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA (46). Oocysts were placed on 4 well glass slides and warmed to 37˚C until dry. The slides were removed from the slide warmer and 15 µl of methanol was added to the oocysts and dried

for 5 minutes. The slides were moved to a container with a paper towel soaked in water to provide humidity. A polyclonal antibody was diluted 1:50, 1:100, 1:200, and 1:500 to determine concentration to be used and an optimum 1:100 dilution was subsequently used. The antibody was then added to the slide as recommended by the supplier, the container was sealed and held for 30 minutes. After 30 minutes, the α-FITC was carefully removed to avoid disturbing the oocysts, then the slides rinsed once with PBS before staining with DAPI. DAPI stock was made by adding 2 mg/mL DAPI to absolute methanol and was stored at 4˚C. The stock DAPI was diluted in water to final concentration of 0.4 mg/ml just prior to use. Slides with DAPI were held at room temperature for 30 minutes, rinsed 3X with PBS, then refrigerated until examined.

Antibody labeling was also done on membrane filters for examination with the Scan*RDI* by placing the membrane with the oocysts directly on 60 µl of methanol for 10 minutes, then transferring the membrane on the filter apparatus and adding 0.5 ml of diluted filtered antibody. A piece of filter paper soaked in water was placed in a 35mm Petri dish and placed on the filter funnel to provide humidity and prevent the antibody from drying out during the incubation time. After 30 minutes in the dark, the antibody was removed with a vacuum pump, the membrane was washed 1X with PBS and 0.5 ml of DAPI placed on the membrane. This was also left on the membrane for 30 minutes as above, removed and the membrane then rinsed 3 times with PBS. The membrane was dried and stored at 4˚C prior to examination.

Polymerase Chain Reaction

PCR was explored as a method to verify the presence of *Cryptosporidium* from water as determined by FISH and FAb. To detect *Cryptosporidium* in small numbers by PCR, a nested PCR approach was used (56, 57). The DNA was extracted from 1X 10^8 *Cryptosporidium* oocysts using the FastDNA Spin kit for soil (MP Biomedical) (58) and the DNA diluted to the equivalent of from 10^4 oocysts to 1 oocyst and stored at -20˚C. Two protocols were tried.

To amplify fragments of the 18S rRNA as reported by Coupe, et. al. (57) initial amplification was performed using SCL1 (5'-CTGGTTGATCCTGCCAGTAG-3'), and CPB-DIAGR (5'TAAGGTGCTGAAGGAGTAAGG-3'). PCR conditions were 5 min at 94°C, followed by 39 cycles of denaturing at 30 s at 94°C, annealing for 45s at 60°C, extension for 90 s at 72°C then 10 min at 72˚C final extension, held at 4˚C. The second-round PCR amplifies a 214-bp fragment interior to the first using SCL2 (5'-CAGTTATAGTTTACTTGATAATC-3') as the forward primer and SCR2 (5'CAATACCCTACCGTCTAAAG-3') as the reverse primer. For 25 µl reaction volume, 0.5 units of LATaq (Takara), 0.2 mM deoxynucleoside triphosphate mix, 0.5 µM each of the primers and 1 µl of the template. The PCR conditions for second-round PCR were the same as the first, except that final volume was 50 µl, the primer concentrations were 0.4 µM, 5 µl of the first amplification product was used as the template, and annealing lasted 45 s at 58°C and extension 60 s at 72°C.

In order to amplify the *Cpgp40/15* gene as described by Ochiai, et. al. (56), the first round PCR was performed with the primer set gp40/15–51 (5'-TCCGCTGTATTCTCAGCCCCA-3') and gp40/15–31 (5'-

AGCAGAGGAACCAGCATCCTT-3'), and the nested PCR, interior to the first fragment, was carried out with gp40/15–52 (5'-TGTTCCTGTTGAGGGCTCATC-3') and gp40/15–32 (5'-GGCAAACAAATCGACGGTTGC-3') (IDT). The first and nested PCR was performed in a 25 µl reaction system of 1x buffer, 0.2 mM deoxynucleoside triphosphate mix, 0.5 µM each of the primers, 0.2 units of ExTaq polymerase (Takara) and 1 µl of template. Extracted DNA was used as template for the first PCR and 1 µl of the product from the first reaction as template for the second. PCR conditions for both the first and nested amplification for the *Cpgp40/15* gene were 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

PCR products were visualized by electrophoresis using 10 µl of amplicon run through a 1.25% agarose gel at 80 volts and stained with ethidium bromide. Images were taken through an ethidium bromide filter on a Fotodyne imaging system.

Imaging and Enumeration

Examination of *Cryptosporidium* oocysts was done as described in previous chapter, using an Axioskop 50 (Zeiss) microscope with a 100X oil objective, imaged with an AxioCam 412 using Axiovision software (Zeiss) for 100 or 150 msecs and the images analyzed with Metamorph software (MetaImaging Systems ver. 7.7) to quantitatively determine the fluorescent intensity of the probed oocysts. The fluorescent intensities that had been generated by the different methods were compared to ascertain improvements in signal intensity resulting from the changes in methodology.

Membranes were enumerated with the Scan*RDI* and development of the FISH specific applications was done as described in the preceding chapter by sorting the each of discrimination factors of all fluorescent events positive for *Cryptosporidium* oocysts in an Excel spreadsheet to provide a range in each of the 11 discrimination factors used by the Scan*RDI*. These ranges were compared to the FISH application that had been developed using *E. coli* labeled with FISH. Additional membranes were examined using the FISH application in the 'validate all' mode to determine if all oocysts labeled by with the FISH probe could be viewed on the results screen of the Scan*RDI*.

Examination of α-FITC labeled oocysts on slides was done using a Nikon Eclipse E800 with filters having an Ex/Em of 480/535, dichroic mirror 505LP for fluorescein, 355/460, dichroic C400 LB for DAPI and differential interference contrast (DIC), which was used to identify internal morphological characteristics in *Cryptosporidium* oocysts. Filter membranes were examined with the Scan*RDI* using the tvc application and validating all.

Results

Permeabilization for FISH

All of the oocysts fixed using 80°C ethanol on membrane filters were poorly labeled, producing an average fluorescent intensity of 8703 as determined using the Metamorph software. The oocysts fixed and permeabilized using the technique developed with *E. coli,* i.e., fixing with 4% paraformaldehyde for 1 hour followed by a series of room temperature ETOH baths, then lysozyme treatment for 10 minutes, had an average

fluorescent intensity of 32,124, adequate for detection by the Scan*RDI* and compatible with the goal of detection of *E. coli* and *Cryptosporidium* on the same filter.

FISH Probe Concentrations and Hybridization

The optimization of FISH probe concentration was done using the hybridization buffer suggested by Deere et.al. (25) without formamide. A final concentration of 1 pmol/µl probe provided the highest average fluorescent intensity, but because the fluorescent intensity produced using 0.5 pmol/µl was sufficient for detection by the *ScanRDI*, in order to conserve probe, subsequent hybridizations were be done at 0.5 pmol/µl concentrations. At 1 pmol/µl and above, the fluorescent intensity was saturated at 65,000 when images were taken at 150 msec, and all subsequent images of FISH labeled oocysts were taken with 100 msec exposure. The number of oocysts enumerated was the same if the probe concentration was between 0.25 and 1.0 pmol/µl. Data are shown in Figure 3.1.

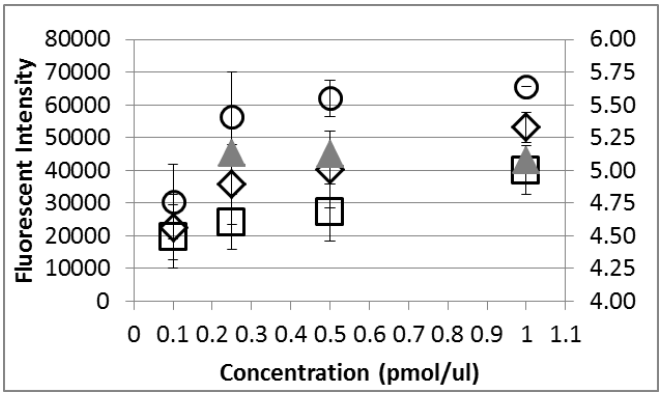

Figure 3.1. Comparison of Fluorescent Intensities Produced by Varying CRY-HRP Probe Concentration. Maximum (circles), mean (diamonds) and minimum (squares) fluorescent intensity of oocysts as determined with the Metamorph software. Log of the number of oocysts represented by triangles n=3.

After probing with CRY-HRP and CRY-Bio using methods above, followed by appropriate TSA protocol, the probe labeled directly with HRP produced an average intensity 55% higher than the probe labeled with biotin. CRY-HRP was used for all remaining *Cryptosporidium* FISH experiments.

Subsequent hybridizations compared incubation temperatures in hybridization buffer with or without formamide. Incubation from 1.5 to 2 hours at 48˚C in a buffer without formamide following permeabilization as described above with a series of ETOH baths and lysozyme treatment, provided the optimum fluorescence and was established as the standard hybridization protocol for *Cryptosporidium*.

Storage of Membranes Containing Labeled Oocysts

Oocysts labeled with FISH could be stored on absorbent media pads for at least 7 days prior to examination with no loss of fluorescent intensity. Storing membranes on 2 ml TNT buffer in an absorbent pad prior to enumeration created increased variability when compared to oocysts read at day zero and membranes stored on absorbent pads with no liquid added. See Figure 3.2.

Detection with Fluorescent Antibodies

As shown in Figure 3.3, various dilutions of the polyclonal goat α-Cry were made to determine which provided the brightest intensities for detection with the Scan*RDI*. It was found that although a 1:50 dilution rendered the brightest oocysts, it gave a high background and 1:00 dilution was used.

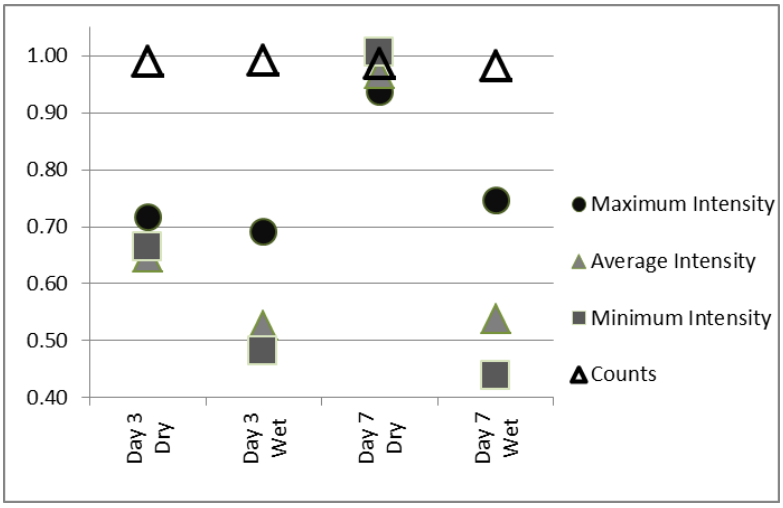

Figure 3.2. Ratio of Fluorescent Intensities of Oocysts Stored at 4˚C
After hybridization with FISH, membranes were stored dry or with TNT buffer (wet). Maximum (circles), mean (diamonds) and minimum (squares) fluorescent intensity of oocysts as determined with the Metamorph software. The log transformed number of oocysts enumerated is represented by triangles n=2.

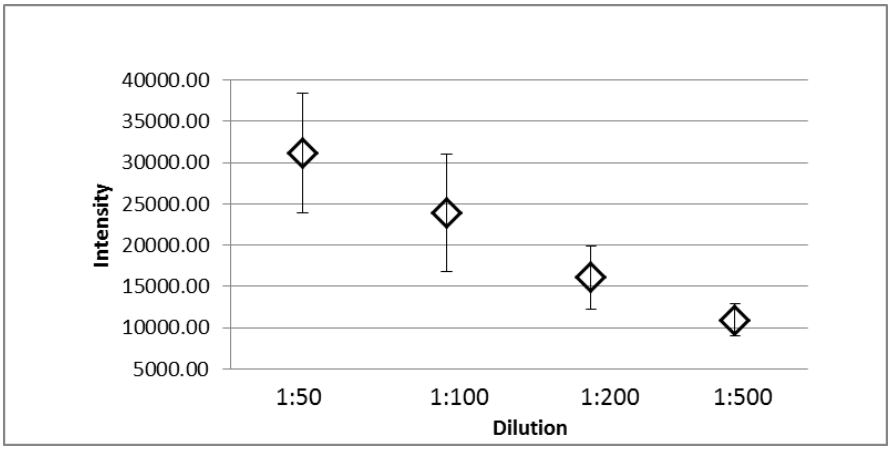

Figure 3.3. Comparison of Fluorescent Intensities Produced by Varying the FAb Concentrations. Average fluorescent intensity of oocysts as determined with the Metamorph software. Images taken with 150 msec exposure n=4.

Attempts were made to use FAb labeled oocysts with the Scan*RDI* in order to be able to compare numbers of oocysts detected by FISH directly with numbers detected by FAb. There were large numbers of false positive fluorescent events due to cross reaction

of the proteinaceous antibody with non-oocysts material on the membrane. The numbers of false positives were between 438 and 7018 events on 17 membranes tested with and without oocysts present, with a mean of 3733 events.

Detection of Control *Cryptosporidium* Oocysts by FISH and FAb

Controls were done using known numbers of *Cryptosporidium* oocysts enumerated with the Scan*RDI* using the FISH protocol described and FAb using the EPA epifluorescent/DIC method. The threshold settings and discriminant settings for the Scan*RDI* are shown in Table 2.4.

Comparison of these controls show considerable variation over the course of the study (Table 3.1); the ratio between oocysts detected by FISH compared to FAb ranged from 0.02 to 2.16 with a median of 0.42. In each case, the oocysts used as controls had been held for several months at 4˚C prior to use. Examination of oocysts by the EPA method showed clumps of oocysts that were counted individually.

Table 3.1. Number of Control Oocysts Detected by the EPA or Scan*RDI*

Date	EPA		ScanRDI	
	#	Log	#	Log
9/9/2011	196	2.29	315	2.50
9/21/2011	109	2.04	235	2.37
2/21/2012	236	2.37	62	1.79
4/13/2012	973	2.99	204	2.31
5/22/2012	45	1.65	26	1.41
6/28/2012	158	2.20	3	0.52
Mean	286		141	

Polymerase Chain Reaction

To determine if PCR could be used to verify the presence of oocysts in water samples, a nested PCR protocol was done. Although the lower detection limit of the nested PCR was reported to be template equivalent one oocyst (56, 57), using either the Coupe protocol (57) or the Ochiai protocol (56) gave faint bands for the equivalent of 10 oocysts only once and 100 oocysts for all remaining attempts. As expected, the first round PCR produced detectable template equivalent to 1000 oocysts.

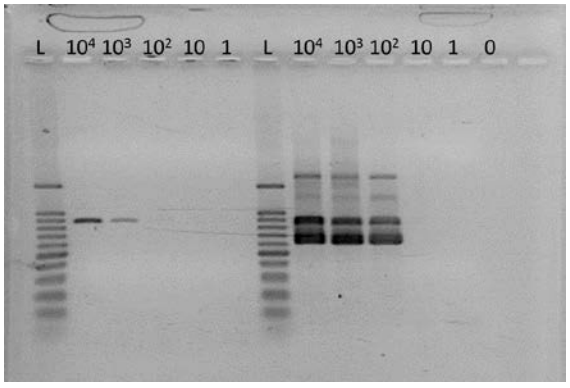

Figure 3.4. Gel Showing Nested PCR Using Ochiai Primers
Lanes 2 through 6 are the product of the first PCR and lanes 8 through 12 show the product of the second PCR. Lanes 1 and 7 are DNA markers.

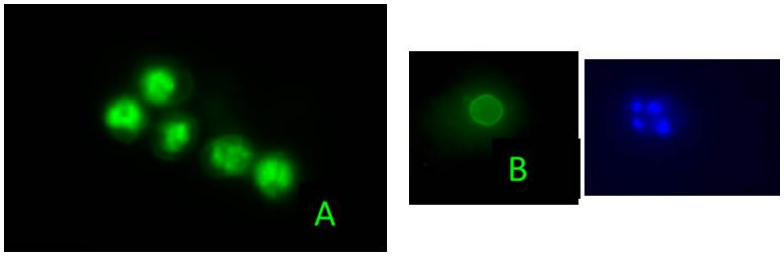

Figure 3.5. *Cryptosporidium* Oocysts Labeled A) with FISH or B) with FAb and DAPI

Discussion

Before attempting detection of *Cryptosporidium* oocysts from the environment, protocol to label *Cryptosporidium* with FISH on a membrane filter and detection using the Scan*RDI* needed to be optimized to provide the brightest, most consistent fluorescent signal possible. In addition, since the number of oocysts detected by the FISH label would be compared to the number found with the FAb label recommended by the EPA, the FAb method needed to be compared to the control oocysts.

The 1:100 dilution of antibody was found to provide adequate fluorescent intensity for detection of *Cryptosporidium* oocysts with the *ScanRDI,* but in every case there were high numbers of labeled debris that prevented proper enumeration. Additionally, DIC examination of the FAb labeled oocysts, as required by the EPA method, could not be done on the membrane filters. It was decided that detection of *Cryptosporidium* should be done using the EPA method on slides with manual enumeration for comparison to the FISH labeled oocysts on membranes enumerated with the Scan*RDI*.

DNA from oocysts was set aside after purification and PCR was to be done after sampling and purification of oocysts from environmental water samples as a verification of the presence of oocysts in the water. Since only 100 oocysts could be detected reliably, this technique was not pursued.

Comparison of the two methods using control oocysts gave variable results, at times the Scan*RDI* method found 2.16 times the number detected by the EPA method and others less than 2% of the number found by the EPA method. The control oocysts were

often clumped together and counted as individual oocysts inflating the number counted by the EPA method while the parameters set by the Scan*RDI* application may have prevented those clumps of oocysts from being detected.

The protocol developed for detection of *E. coli* by FISH and the Scan*RDI* (reported in previous chapter), also allowed detection of known *Cryptosporidium parvum* oocysts. The optimal fluorescent signal was obtained after paraformaldehyde fixation, dehydration with an ethanol series and permeabilization with lysozyme. The enzyme, lysozyme, is reported to break down the peptidoglycan in bacteria by breaking the β-(1,4) glycosidic bond between *N*-acetyl muramic acid and *N*-acetyl glucosamine, which increases the permeability allowing the probe plus HRP access to the cell (59, 60). From these results it is apparent that the oocyst wall is also acted on by lysozyme, allowing the probe plus HRP through the oocyst wall and enabling the labeling the sporozoites. Probe used at 0.5 pmol/µl was adequate for detection of oocysts with the Scan*RDI* and hybridization time between 1 and 2.5 hours provided a bright and reliable method for labeling of the oocysts.

Development of a reliable technique for labeling *Cryptosporidium* oocysts with FISH on membrane filters provides a method that can be used to detect *Cryptosporidium* with Solid Phase Laser Cytometry. The following chapter describes the use of this method on environmental samples collected from river and treated drinking waters.

DETECTION OF *CRYPTOSPORIDIUM* FROM THE ENVIRONMENT

Introduction

C. parvum, associated with cattle and other livestock , and *C. hominis,* associated with mainly with human or non-human primates, are the primary causes of disease in humans (61). It has been estimated that cattle can shed 10^7 or more oocysts/animal/day (62, 63).

Cryptosporidium oocysts are resistant to disinfectants such as chlorine and monochloramine in concentrations commonly used to treat drinking and waste water. They remain viable in water during disinfection by 80 ppm chlorine for 90 minutes (64) and, as drinking water is commonly treated with 2 mg/l free chlorine, the conditions found in normally functioning drinking water treatment plants will not prevent contamination of the distribution system by viable *Cryptosporidium* that may be present in source water. Once in the drinking water system, it is also possible that the oocysts can associate with biofilms, later detaching in high enough numbers to provide an infectious dose (65) . The importance of drinking water as a source of infection was clearly demonstrated in 1993, during an outbreak in Milwaukee, WI when over 400,000 people were infected as oocysts passed through the city's filtration and chlorination system and into the distribution system (37, 66).

The infective dose in healthy individuals has been reported to be from 10 to 132 oocysts, dependent on the strain (42, 44), although it has been extrapolated from outbreaks that certain people could develop the disease following ingestion of only one oocyst (37).

Because *Cryptosporidium* oocysts are more resistant to degradation in the environment than common indicators of fecal contamination such as *E. coli*, oocysts may be present when no *E. coli* cells are found (67-70).

In view of the large numbers of oocysts shed by infected animals, the range of *Cryptosporidium* species that can infect humans, and the low infectious dose needed to cause disease even in healthy populations, it is important to the monitor the environments where transmission of *Cryptosporidium* to humans may occur. The most common form of transmission is through water, either directly through ingestion of water contaminated with oocysts, or indirectly through food that has been irrigated, sprayed or washed with that water. It has been estimated that up to 96% of surface water may be contaminated with some *Cryptosporidium* spp.(71). In view of these, monitoring water for *Cryptosporidium* oocysts seems the most likely means to have the largest effect on the public health. To assure drinking water is free from infective oocysts, a rapid method for direct detection of *Cryptosporidium parvum* in source water would be valuable.

Because analyzing for and detecting all possible waterborne pathogens is difficult, detection of fecal contamination in water through the presence of indicator bacteria including fecal coliforms, enterococci, and *Escherichia coli* has been the EPA standard since 1986 (72). Although some studies have shown a strong correlation between the presence of *E. coli* and *Cryptosporidium* (68), *Cryptosporidium* are much more resistant to degradation in the environment than bacteria, and it remains likely that viable oocysts could remain in water after fecal indicators are no longer detectable. Wilkes, et.al.(68), found that in agricultural water, only 39-43% of water containing *Cryptosporidium* had indicator bacteria (68). Many reports with findings of *Cryptosporidium* as the cause of

disease outbreaks have not demonstrated the presence of fecal indicator bacteria (69, 70, 73). A rapid, reliable detection method that could be used when *Cryptosporidium* is likely to be present, even in the absence of indicator bacteria, would be beneficial.

The current method recommended by the EPA for detection of *Cryptosporidium* from water is by epifluorescent microscopy following labeling of oocysts with a fluorescent antibody (FAb). This is coupled with DAPI staining for visualization of the nuclei of sporozoites inside the oocyst wall and differential interference contrast microscopy for examination of internal structures (46). This method relies on manual examination of stained slides. Fluorescent antibodies are not specific to *Cryptosporidium parvum* and may label other non-infective *Cryptosporidium* species (74) as well as non-*Cryptosporidium* material, for instance, the FAb product Aqua-Glo (Waterborne) insert states that their antibody may cross react with certain algal cells. Fluorescent *in situ* hybridization (FISH) targeted to the 18S rRNA, which has been used as a both a more specific detection method and also a verification of viability in *Cryptosporidium parvum* from the environment (25, 37, 49, 52, 53, 74, 75), has thus far also required manual examination on membrane filters or slides.

As discussed in the previous chapters, this study uses the Scan*RDI* (AES Chemunex), a solid-phase laser scanner, that is able to scan the entire surface of a 25 mm diameter membrane filter using a 488-nm laser (4, 15-18) for detection of FISH probed organisms. A new Scan*RDI* application was developed for use with FISH probed organisms. This chapter describes how combining the specificity and the verification of viability through FISH labeling of *Cryptosporidium parvum* and the low detection limit

of the *ScanRDI* provided a rapid method for detection of *Cryptosporidium* from environmental samples.

The Little Big Horn River is used as source water for the drinking water treatment plant at Crow Agency, Montana. Water treatment consists of flocculation, sand/anthracite filtration, and chlorination. The Little Big Horn River flows north through the Crow Indian Reservation, past a confined feeding operation, numerous smaller ranches and rural housing before entering the treatment plant. This provides ample opportunity for contamination of the water with *Cryptosporidium* and other pathogens through run-off into the river. The Scan*RDI* was used for detection of *Cryptosporidium* oocysts labeled with FISH from both the Little Big Horn River source water as well as treated water and those results were compared to the number of *Cryptosporidium* identified by the standard EPA method.

Methods

Organisms, Oligonucleotide Probes, and Antibodies

Cryptosporidium parvum oocysts were obtained from Sterling Parasitology Laboratory at the University of Arizona shipped in antibiotics at 1 x 10^8 oocysts/ml. Oocysts were stored at 4˚C for up to 4 months.

The oligonucleotide probe used to identify *Cryptosporidium parvum*, CRY-1, (49), was purchased labeled with horse radish peroxidase (CRY-HRP). A non-eubacterial probe, NONEUB, (22), or the ECO541 probe, (12), both labeled with HRP, were used as a negative controls to probe for *Cryptosporidium*. All HRP labeled probes were

purchased from biomers.net or Thermo Scientific. Upon receipt, all probes were hydrated in water to 100 pmol/µl, aliquoted into 20 µl amounts and stored at -20˚C.

For examination of environmental samples using the EPA procedure (46), AquaGlo, a fluorescein labeled α-*Cryptosporidium* antibody was obtained from Waterborne. It was used and stored according to manufacturer's directions.

Sample Collection and Concentration of Oocysts

Water was sampled at the Crow Agency, Montana, water treatment plant on the Crow Indian Reservation. Source water for the treatment plant is the Little Big Horn River. Water was collected and samples processed using guidelines from EPA protocol (46) as follows. Ten to 20 liters of water were filtered through a Filta-Max foam filter apparatus (Idexx) using water drawn from the post-treatment valve or an intake valve at the water treatment plant. Post treatment samples were taken first to assure there would be no cross contamination with source water. Flow rate was measured prior to and during collection to verify flow rate was 2 l/min. Temperature and pH of the water were measured and turbidity data was provided by the water treatment plant operators using the Hach turbidity meter. Chlorine concentration of the treated water was measured. After filtration, filters were placed in sealable plastic bags and stored in a cooler between 1 and 10˚C until return to the lab. Additional water from each source was collected in 1 liter sterile plastic bottles and placed in the cooler. Nine samples were taken over the course of one year, from June 2011 through June 2012.

Heterotrophic Plate Counts, Coliforms and *E. coli*

Water samples were returned to the lab within 8 hours after collection, stored at 4˚C and processed within 15 hours after receipt.

Water was filtered through 0.45 µm pore sized 47 mm gridded mixed cellulose filter membranes (Millipore) in 100 ml volumes for treated water and 1, 10 and 100 ml volumes for source water X 3. Membranes were placed on 2 ml of mColiBlue® (Hach) and incubated 24 hours at 36˚C. After incubation, membranes were enumerated using a dissecting microscope and red colonies were enumerated as coliforms other than *E. coli* and blue colonies were counted as *E. coli* and the number recorded in CFU/100 ml.

Heterotrophic plate counts were done by diluting water as necessary to obtain between 20 and 200 CFU per plate and spread plating on R2A agar. The plates were incubated for 7 days at 30˚C prior to enumeration. Counts were reported in CFU/ml.

Elution of Oocysts by Stomaching

Oocysts were removed from the Filta-Max filters using the stomacher method. This was done by placing the filter in a 4 X 6 inch Stomacher 80 bag (Seward), removing the bolt from the filter module and separating the foam rings into two portions. Half of the foam filters were placed in a second stomacher bag. The filter caps were removed from the stomacher bag and rinsed with phosphate buffered saline with Tween 20 (PBST; NaCl 8g, 0.2 g KCl, 1.15 g Na_2PO_4 anhydrous and 0.2 g KH_2PO_4, 100 µl Tween 20 per liter of water pH adjusted to 7.4) (46) Three hundred milliliters of PBST were added to each stomacher bag containing the filter pads and bags were allowed to sit for 5 minutes to allow expansion of the foam. Bags were placed serially into the stomacher (Seward)

and washed for 5 minutes on a normal setting. After stomaching, the PBST was poured into a 2 liter beaker. Another 300 ml volume was added to each of the bags and stomaching repeated. This PBST was added to previous volume. The bags containing the foam filters were wrung out and any remaining PBST added to the previous volume. The foam filters were discarded and bag was then rinsed with a small amount of PBST which was also added to the total volume.

Concentration of Eluted Oocysts by Centrifugation

The total pooled volume from the eluate in the 2 liter beaker was measured into 250 ml centrifuge bottles and centrifuged for 15 min at 3500 X G (Sorval GSA Rotor). The supernatant was aspirated using a sterile Pasteur pipet to 5 ml above the pellet. The pellet was mixed thoroughly with the remaining 5 ml volume to resuspend and all pellets pooled into a 30 ml centrifuge tube. The bottles were rinsed and rinse was added to the centrifuge tube. This volume was concentrated by centrifugation at 3500 X G for 15 min (Sorval SS-34 rotor). Supernatant was removed as described previously and pellet volume was recorded.

Purification Using Immunomagnetic Separation

After centrifugation, immunomagnetic separation (IMS) was used to separate oocysts from particulates. The total volume of pellet was used when examining environmental samples, pellet volume was adjusted to give ≤ 0.5 ml pellet/ in each sample as described by the EPA protocol (46). The pellet was thoroughly vortexed to resuspend in the material water and divided when necessary as described by the EPA protocol. IMS was done using the Dynal *Cryptosporidium* BeadRetriever System Kit

(Idexx) following manufacturer's instructions. Briefly this includes addition of buffers and paramagnetic beads to tubes with samples and rotation at room temperature for 1 hour to allow oocysts to adhere to antibodies attached to beads. Magnets were used to draw paramagnetic beads from the sample, followed by repeated rinsing, resuspension of beads and magnetic removal of beads from suspension.

After purification, oocysts were removed from the beads using repeated application of HCl, the pH was neutralized and all oocysts from one sample were pooled into a single volume. Equal sample volumes were used for FISH and FAb, these volumes were divided so samples could be processed in duplicate.

Fluorescent *in situ* Hybridization

For positive controls, oocysts were diluted to 1 x 10^3 oocysts/ml then 100 µl of the suspension were filtered through the center of a black 0.45 µm pore-sized polyester membrane (AES Chemunex) to give approximately 100 oocysts/membrane filter. For environmental samples, aliquots of the pooled volume of IMS purified oocysts were filtered as above. Fixation, permeabilization and hybridization were done using methods adapted from Baudart et. al, and Lepeuple, et. al, (6, 7, 14) and described in the previous two chapters. Controls were done using known membranes containing known numbers of oocysts and water with no oocysts as negative controls.

Fluorescent Antibody Labeling and Plate Counts

Oocysts were identified with fluorescein labeled α-*Cryptosporidium* antibodies (α-FITC) as described in EPA Method 1622: *Cryptosporidium* in Water by

Filtration/IMS/FA (46). Controls were done using known numbers of oocysts and appropriate negative controls.

Imaging and Enumeration

Membranes containing oocysts were enumerated with the Scan*RDI* mounted onto the specially designed Scan*RDI* holder and scanned as described above. The membranes were analyzed using the FISH software application. Frequency thresholding and discriminant settings are shown in Table 2.1.

Examination of α-FITC/DAPI labeled oocysts on slides was done as described in the preceding chapter with epifluorescent microscopy and differential interference contrast (DIC).

Calculations

The number of oocysts in the environmental water sample was calculated based on the total volume of water filtered, the volume of pooled sample after purification, and the volume that was used for each detection method. Not all of the purified volume was used for examination by either method (see Table 4.1).

Table 4.1. Formulae for Determining Oocyst Numbers

To determine the number of oocysts detected by each of the two methods this formula was used:

$$\text{\# of oocysts/liter} = \frac{\text{\# oocysts detected by FISH or FAb} \times \frac{\text{Total purified volume (µl)}}{\text{Volume examined per method (µl)}}}{\text{\# liters collected}}$$

To obtain the total number of oocysts per liter of water collected this formula was used:

$$\text{Total \# of oocysts/liter} = \frac{\text{Total \# oocysts detected by both methods} \times \frac{\text{Total purified volume (µl)}}{\text{Total volume examined (µl)}}}{\text{\# liters collected}}$$

Results

Detection of *Cryptosporidium* from Source Water

Cryptosporidium oocysts were found by both the FISH/Scan*RDI* and EPA/FAb methods in every sample taken from the Little Big Horn River. The number detected fluctuated throughout the year, with the highest concentrations found in June (30.7 oocysts/liter) and July, 2011 (80.9 oocysts/liter) and the lowest concentrations in September (0.3 oocysts/liter) and October (0.5 oocysts/liter) the same year, regardless of sampling method (Table 4.2).

Also shown in Table 4.2 are the numbers of *E. coli* found in the Little Big Horn River. These exceeded the EPA recommendation of 126 CFU/100 ml for recreational water on 4 occasions (72). A paired t-test was used to compare log transformed results from FISH and FAB oocyst counts from the Little Big Horn River sampling to show that .significantly more oocysts were detected using the Scan*RDI* following FISH probing compared to detection with FAb plus DAPI with epifluorescent microscopy and DIC in all the samples taken from the Little Big Horn River ($p = 0.005$) (see Figure 4.1).

Table 4.2. Results of Sampling the Source Water from the Little Big Horn River The column labeled EPA shows oocysts/liter as detected by EPA method FAb/DAPI and DIC and the column labeled Scan*RDI* shows oocysts/liter as detected by the Scan*RDI* after FISH probing with CRY-HRP. *Scan*RDI* result lost due to lab error.

Date	Coliform (CFU/100 ml)	E. coli (CFU/100ml)	Oocysts EPA #/liter	Oocysts Scan*RDI* #/liter	Total oocysts in Sample (Calculated)	Total oocysts/liter (Calculated)
6/17/2011	6130	267	16.6	44.9	461.1	30.7
7/8/2011	6700	130	70.0	91.7	1212.8	80.9
8/10/2011	5230	333	2.4	ND*	24.0	1.2
9/8/2011	7667	23	0.1	0.6	6.8	0.3
10/27/2011	630	13	0.4	0.5	9.56	0.5
2/22/2012	1313	10	2.5	3.1	27.9	2.8
4/13/2012	597	50	1.4	5.7	30.0	3.0
5/22/2012	2800	370	3.7	8.2	51.3	6.0
6/28/2012	1537	63	0.4	0.7	8.0	0.5

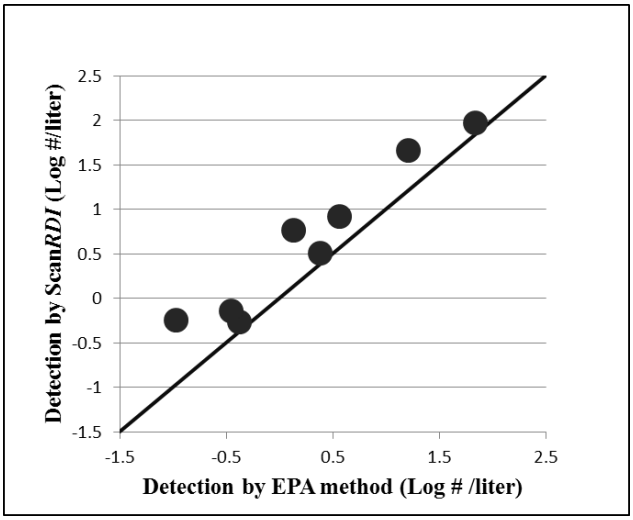

Figure 4.1. Comparison of Oocysts Detected by FISH/ScanRDI and Oocysts Detected by FAb/DAPI/DIC Line represents the line of equality

Correlation between *E. coli* and *Cryptosporidium* Counts

There was no relationship between the numbers of *Cryptosporidium* oocysts to either *E. coli* or coliform counts as determined by mColiBlue®, in water taken from the

Little Big Horn River (Figure 4.2). Sometimes *E. coli* counts were above the EPA recommendation of 126 CFU/100 ml in recreational water when *Cryptosporidium* counts were lower and sometimes below the recommended limit when *Cryptosporidium* were higher.

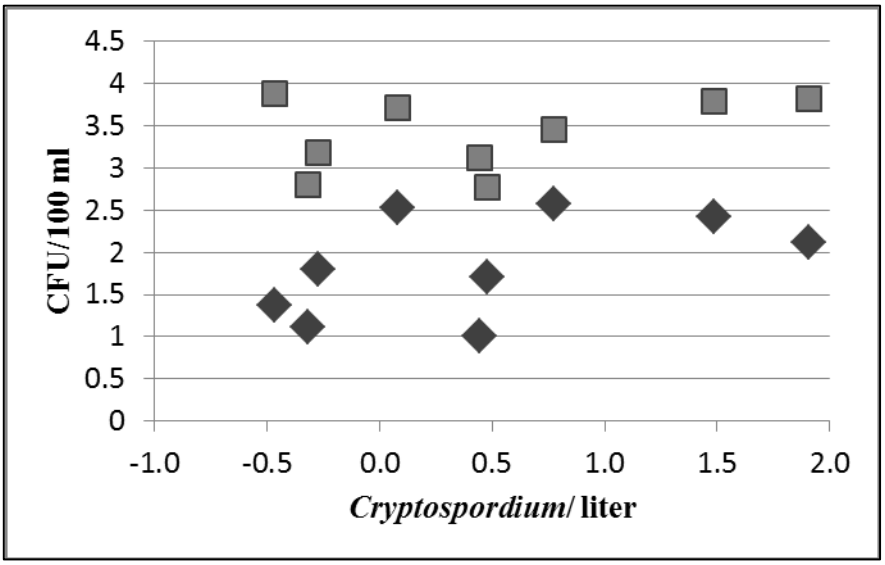

Figure 4.2 Number of *Cryptosporidium* Oocysts Compared to the Number of *E. coli* and Coliforms from the Little Big Horn River
Cryptosporidium numbers shown in log oocysts/liter and *E. coli* and coliform counts shown in log CFU/100 ml. Squares represent coliform counts and diamonds represent *E. coli* counts. Statistics were done using separate regression analyses to estimate the correlation between each organism and statistical significance was assessed with an F-test. It was determined there was no correlation between the number of *E. coli* or coliforms from mColiBlue counts and the number of *Cryptosporidium* oocysts from in the Little Big Horn River. $r^2 = 25.9$, p=0.161 with a correlation of 0.5089 for *E. coli* to *Cryptosporidium* and $r^2 = 13.9$, p= 0.324 with a correlation of 1.6 for coliforms to *Cryptosporidium.*

Detection of *Cryptosporidium* in Treated Water

In treated water, no *E. coli* or coliforms were detected at any sampling point, but in June and July 2011, the drinking water samples contained *Cryptosporidium parvum*

oocysts. In the July sample oocysts were found by both EPA (1.10/liter) and Scan*RDI* (0.18/liter) methods, but in the June sample, oocysts were only detected with the Scan*RDI* method (1.7 oocysts/liter). (see Table 4.3).

Table 4.3 Results of Sampling the Treated Water from the Treatment Plant EPA designates all oocysts detected by EPA method FAb/DAPI and DIC. Scan*RDI* designates all oocysts detected by the Scan*RDI* after FISH probing with CYR-HRP.
* Not available Scan*RDI* result lost due to lab error.

Date	Coliforms (CFU/100 ml)	E. coli (CFU/100 ml)	Oocysts EPA #/liter	Oocysts Scan*RDI* #/liter	Total oocysts in Sample (Calculated)	Total oocysts/liter (Calculated)
6/17/2011	Below Detection	Below Detection	0.0	1.7	34.0	0.8
7/8/2011	Below Detection	Below Detection	1.1	0.2	12.8	0.3
8/10/2011	Below Detection	Below Detection	None found	NA*	None found	None found
9/8/2010	Below Detection	Below Detection	None found	None found	None found	None found
10/27/2011	Below Detection	Below Detection	None found	None found	None found	None found
2/22/2012	Below Detection	Below Detection	None found	None found	None found	None found
4/13/2012	Below Detection	Below Detection	None found	None found	None found	None found
5/22/2012	Below Detection	Below Detection	None found	None found	None found	None found
6/28/2012	Below Detection	Below Detection	None found	None found	None found	None found

Discussion

We have found that use of the Scan*RDI* after fluorescent *in situ* hybridization using CRY-HRP followed by tyramide signal amplification will provide better detection of *Cryptosporidium* oocysts from environmental water compared to the EPA method of FAb/DAPI and DIC (Figure 3.1). The source water for the water treatment facility at Crow Agency, Montana, is the Little Big Horn River which may be contaminated by both

cattle operations and rural houses upstream from the treatment plant. *Cryptosporidium parvum* oocysts were found in all river water samples tested by both EPA and Scan*RDI* methods, with highest numbers found in June and July, 2012. In May, 2012, there was a major flood that carried substantial runoff into the river and also brought river water directly into the treatment plant, preventing sampling during that month. Following this flood event, no coliforms or *E. coli* were found in the treated water. However, *Cryptosporidium parvum* oocysts were found using the Scan*RDI* method in the treated water in June and by both methods in July. By August and during the remainder of the sampling period, no oocysts were found in the treated water by either method. There was no correlation found between the number of *E. coli* or coliforms and the number of *Cryptosporidium* from either the treated or source water.

After use of the EPA method for concentration and purification, examination of source water and treated drinking water for *Cryptosporidium* oocysts using FISH probes and the Scan*RDI* allowed detection of more *Cryptosporidium* oocysts than the EPA method which relies on the FAb/DAPI/DIC. This method was less likely to cause rinse-off of oocysts during labeling and after labeling, it was also faster, more efficient and less prone to error due to technician fatigue.

DETECTION OF *CRYPTOSPORIDIUM* AND *E. COLI*

Introduction

The FISH/Scan*RDI* method was tested for simultaneous detection of *Cryptosporidium* and *E. coli* on the same membrane filter. A method that could be used to identify multiple organisms from water would improve the ability to examine and maintain the microbiological quality of water.

The FISH probe Colinsitu-HRP along with unlabeled helper probes described in the chapter 'Fluorescent *in situ* Hybridization of *E. coli*" for labeling *E. coli*', and the CRY-HRP probe for *Cryptosporidium* were used in hybridization buffer with membranes containing either or both of these target organisms and the number of each determined with the Scan*RDI*. These results were compared to plate counts results for *E. coli* and FAb/EPA results for *Cryptosporidium*.

Methods

Organisms and Probes Used

E. coli was grown overnight at 37˚C on R2A agar from a frozen stock of an environmental isolate from a drinking water distribution system, provided by D. Smith, South Central Connecticut Regional Water Authority, New Haven, Connecticut. *Aeromonas hydrophila*, from the same source, was also grown on R2A as above and used as a negative control. *Cryptosporidium parvum* oocysts were obtained from Sterling laboratories.

The oligonucleotide probe used for *Cryptosporidium parvum* was CRY-1 (49) labeled with HRP (CRY-HRP). For detection of *E. coli*, the Colinsitu probe, (11) labeled with HRP (COL-HRP) was used with helper probes HColin_R, and HColin_L, (7, 14). All HRP labeled probes were purchased from biomers.net. Unlabeled helper probes were obtained from Integrated DNA Technology.

Preparation and Filtration

Oocysts were diluted to 1 x 10^3 oocysts/ml then 100 µl of the suspension were filtered through the center of a black 0.45 µm pore-sized polyester CB04 membrane (AES Chemunex) to give approximately 100 oocysts/membrane filter. A suspension of *E. coli* cells was diluted to 1 x 10^3 cells/ml as described in 'Fluorescent *in situ* Hybridization of E. coli" for labeling E. coli', and 100 µl were filtered. For membranes containing both *E. coli* and *Cryptosporidium* suspensions of 10^3 oocysts/ml and 10^3 bacterial cells/ml were combined and 200 µl were filtered as above.

Hybridization Conditions

The prefixation incubation step was done by placing membranes on a 25 mm absorbent pad (Millipore AP1002500) containing 0.65 µl of R2A broth with nalidixic acid (Acros 227920250) at a final concentration of 100 µg/ml at 37˚C for 4 hours.

Membranes were fixed and permeabilized as previously described and hybridization was done for 1.5 hours at 48˚C, followed by washing, rinse and TSA as described in previous chapters.

Cryptosporidium numbers enumerated with the FISH/Scan*RDI* method were compared to oocysts counts after labeling according to the EPA method with

FAb/DAPI/DIC described in the preceding chapter. For comparison with Scan*RDI* counts for bacterial cells, the suspensions of *E. coli* and *Aeromonas* were plated on R2A agar, incubated at 37˚C overnight and recorded as CFU/100 µl.

Results

Detection of *Cryptosporidium* and *E. coli* Together

Membranes with either *E. coli*, *Aeromonas hydrophila*, *Cryptosporidium* or combinations of *E. coli* and *Cryptosporidium* were incubated for 4 hours prior to fixation and hybridization and examined with the Scan*RDI*. When *E. coli* was probed with CRY-HRP or *Cryptosporidium* was probed with COL-HRP plus helper probes, no cells or oocysts were detected. When *Aeromonas* was probed with CRY-HRP and COL-HRP plus helpers, no cells were seen. Membranes containing *E. coli* probed with FISH and scanned with the solid phase laser cytometry detected approximately76% of cells enumerated by plate counts regardless of whether or not *Cryptosporidium* and CRY-HRP was present (Table 5.1). A 1 sample t-test used to compare log transformed data of *E. coli* probed with COL-HRP to log CFU as determined by plate counts demonstrated that there was no significant difference between any of these results ($p = 0.084$).

When comparisons were made using data from membranes containing *Cryptosporidium* and *E. coli* and both CRY-HRP and COL-HRP plus helper probes in the hybridization buffer, the results were similar.

Table 5.1. Ratio of *E. coli* Detected by Scan*RDI* to Plate Counts when *Cryptosporidium* was present. *E. coli* was probed with COL-HRP with unlabeled helper probes with *E. coli* only on the membrane or a combination of COL-HRP plus helpers and CRY-HRP with *Cryptosporidium* present on the membrane.

	Mean	StDev
E. coli COL	0.77	0.11
E. coli COL & CRY	0.76	0.49

Figure 5.1 shows a comparison between the numbers of oocysts found when *Cryptosporidium* was probed with CRY-HRP or CRY-HRP when COL-HRP and helpers was in the hybridization buffer and was *E. coli* present on the membrane compared to the number of oocysts found using the EPA FAb method. There was no significant difference between these results ($p = 0.3448$). After prefixation incubation for 4 hours, the numbers of oocysts detected by the Scan*RDI* method was improved over detection without prefixation incubation.

Prefixation Incubation of *Cryptosporidium*

Oocysts that had been stored for 4 months then subjected to a prefixation incubation of 4 hours on R2A broth with nalidixic acid were found to have a ratio between 0.63 to 11.27 with a median of 2.08 oocyst detection by the Scan*RDI* over detection with the EPA method. When compared to the same ratio without prefixation incubation the ratio ranged from 0.02 to 2.16 with a median of 0.42 (see Table 3.1).

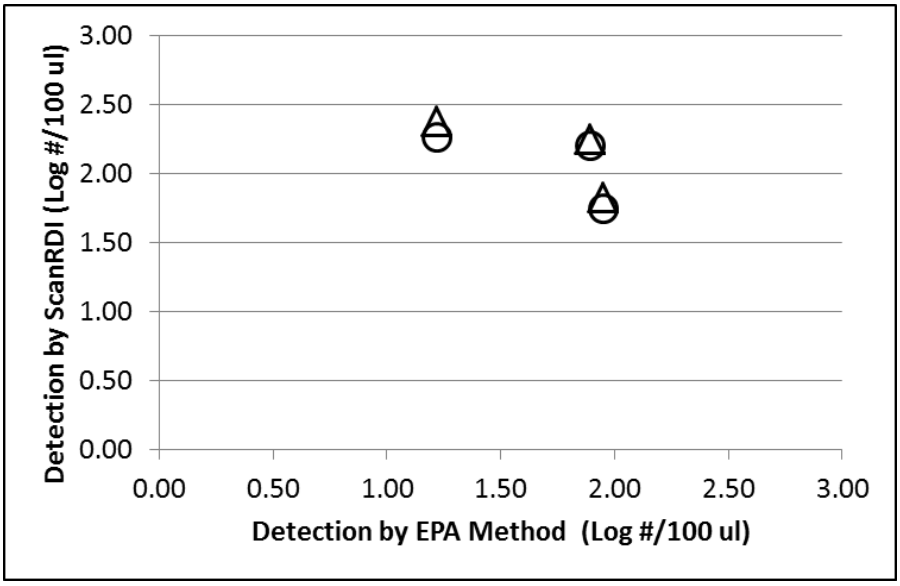

Figure 5.1. Comparison of Oocysts Detected by EPA method compared to ScanRDI/FISH with E.coli-COL-HRP Present
Circles show log of number detected results when only *Cryptosporidium* was present on a membrane labeled with CRY-HRP. Triangles represent the log of the number of *Cryptosporidium* detected with FISH using a combination of CRY-HRP and COL-HRP with helper probes when *E. coli* was also present on the membrane. An ANOVA was fit to the log transformed data with a random effect due to experiment and a fixed effect due to method in order to determine if there was interference or enhancement due to presence of both COL-HRP and CRY-HRP probes and/or presence of both *E. coli* and *Cryptosporidium* on the same membrane.

Discussion

Simultaneous detection of multiple organisms with one rapid detection method would enable examination of drinking water, not just for bacterial indicators of fecal contamination, indicating the possibility of presence of disease causing organisms, but the presence of the pathogens themselves. In this instance, we used *E. coli,* an indicator of the presence of fecal contamination, on the same membrane with *Cryptosporidium parvum*, a human pathogen, to determine if these two organisms could be FISH probed together and then detected using the Scan*RDI*. *E. coli* counts on membranes without

Cryptosporidium and CRY-HRP did not differ from counts of *E. coli* on membranes with *Cryptosporidium* and the *Cryptosporidium* probe. Likewise, the counts of *Cryptosporidium* were not significantly different when *E. coli* and the *E. coli* probe were present. This shows that there was no inhibition or enhancement due to the presence of the other probe or organism.

Additionally, *Cryptosporidium* fluorescence and detection improved after incubation at 37˚C for 4 hours on R2A with nalidixic acid, a condition which was required for optimal detection of *E. coli* using FISH with the Scan*RDI* (7). *Cryptosporidium* oocysts, like bacterial cells, may benefit from a prefixation incubation, especially when they have been in the environment for several months. These two organisms were easy to differentiate based on morphology (see Figure 5.2).

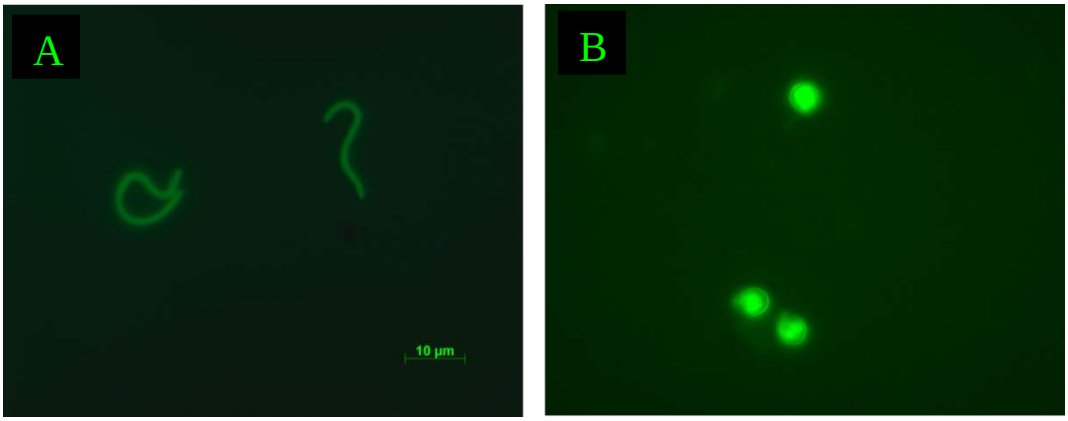

Figure 5.2. A) *E. coli* cells and B) *Cryptosporidium* Oocysts after 4 hour incubation on R2A broth with nalidixic acid

CONCLUSIONS

Use of the solid phase laser cytometry for detection of specific organisms using FISH has been documented (6-8, 14) and identification and viability of *Cryptosporidium parvum* oocysts using FISH has been well established (25, 49, 51-53, 74). This method combines the use of the Scan*RDI* and fluorescent *in situ* hybridization along with the EPA method 1622 for collection and concentration of oocysts using IMS (46).

Preparation of slides for FAb/DAPI is a relatively simple procedure, but examination of samples for *Cryptosporidium* using the EPA method of FAb/DAPI/DIC is a laborious process. Preparation of membranes with FISH is more time consuming than the FAB/DAPI/DIC slide method, but examination of membranes after probing, using the *ScanRDI*, is much faster, with less opportunity for operator error; the scan being completed in 3 minutes and the verification taking between 5 and 30 minutes, depending on the number of extraneous debris on the membrane. On the other hand, if no Scan*RDI* is available, FISH labeling of *Cryptosporidium* could be done in suspension or directly on a microscope slide (25, 49, 52, 53) and because FISH labeled oocysts are viable (49, 74), FISH labeling of *Cryptosporidium* oocysts could replace the FAb/DAPI/DIC labeling and manual examination now recommended by the EPA. Not only is the viability of the oocysts determined along with identification, but because the FISH probe is more specific, there would be fewer labeled particles that made manually scanning the slides so time consuming.

Finding *Cryptosporidium* oocysts in the treated drinking water from the Crow Water Treatment Plant when no *E. coli* were found clearly demonstrates the need for

developing methods that monitor drinking water directly for pathogens especially after perturbation of the water treatment facilities. Because the breakthrough occurred when the oocyst concentration was high (30 to 80 oocysts/liter) monitoring the source water for high numbers of oocysts might prevent contamination of the drinking water.

Simultaneous detection of multiple organisms with one rapid detection method would enable examination of drinking water not just for bacterial indicators of fecal contamination but the presence of the pathogens themselves. Filtering *E. coli* onto the same membrane as *Cryptosporidium parvum*, we determined that these two organisms could be FISH probed then detected simultaneously using the Scan*RDI*. The elongation of the *E. coli* cells after the 4 hour incubation with nalidixic acid provides information about the viability of the bacterial cells (76) and FISH probing shows viability of *Cryptosporidium* (49, 74). By this method, then, we identify and verify the viability of these two organisms at the same time.

While there was no difficulty in microscopically differentiating between *E. coli* and *Cryptosporidium* based on their morphology, if pathogens with similar morphology were found in water, a secondary detection method would be necessary to differentiate between them. This could include use of a probe labeled with a different fluorophore or PCR after microscopic examination of the membrane.

Unfortunately, the presence of autofluorescent material in both river water and drinking water samples necessitated the use of the EPA filtration, concentration and purification procedures before membranes could be efficiently probed and scanned for oocysts with the Scan*RDI*. The problems with concentrating these organisms from the environment without this preparation by directly filtering water samples lay in the

quantity of debris on the membranes. Filtration of even 100 ml of clear treated water sometimes produced over 10,000 fluorescent events that were detectable with the Scan*RDI* FISH application. In this study, the number of *Cryptosporidium* oocysts was never more than 0.08 oocyst per 100 ml of treated water, so these *Cryptosporidium* positive samples would likely have been reported as falsely negative if only 100 ml had been filtered. The Filta-Max filters used for concentrating oocysts is not manufactured to retain bacterial cells, therefore a method that allows concentration of all pathogens to be identified from large volumes of water without also concentrating debris needs to be developed.

One possibility is using the less specific IMS antibody technique for concentration and FISH probing for identification. The addition of large volumes of paramagnetic beads to liters of water would be cost prohibitive. Instrumentation that allows flow of water past immobile antibody-labeled paramagnetic beads similar to the Pathatrix® instrument (Life Technologies), an instrument used for detection of pathogens from food, may provide an answer to this problem. This would eliminate the difficulty of concentration of particles along with pathogens which is a problem with filtration methods.

The Scan*RDI* can be used with FISH as a more efficient method for detection of *Cryptosporidium* oocysts from environmental water samples after purification using the EPA concentration methods. It has the added benefit of providing information about the viability of the oocysts. It was also shown that *E. coli* and *Cryptosporidium* can be detected and differentiated on the same membrane using these methods. When the problems with concentration and purification of other pathogens are overcome,

fluorescent *in situ* hybridization together with the Scan*RDI* could provide a quick and accurate method for detection of several pathogens at once.

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